

1979

The expression of the [Bgl] genetic complex during preimplantation mouse embryogenesis and in ovulated eggs

Steven Esworthy
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THE EXPRESSION OF THE (BGL) GENETIC COMPLEX
DURING PREIMPLANTATION MOUSE EMBRYOGENESIS
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The expression of the [Bg1] genetic complex during preimplantation
mouse embryogenesis and in ovulated eggs

by

Steven Esworthy

A Dissertation Submitted to the
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The Requirements for the Degree of
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ABBREVIATIONS AND DEFINITIONS

B6 - inbred mouse strain C57B1/6J

B6.CBA - a hybrid mouse line with approximately six generations of in-breeding. The line has most of its genome (98%) from B6 and 2% of its genome from CBA/J. The CBA/J genome has been selected for so that the hybrid line contains the [Bgl] locus from CBA/J which has the structural gene for β -galactosidase

B10.C - a congenic inbred strain with the C57B6/10J genetic background and the [Bgl] region of chromosome nine from the strain BALB/cJ

[Bgl] - a genetic locus on chromosome nine of the mouse containing the structural gene and regulatory genes for β -galactosidase expression

Bgl-e - β -galactosidase electrophoresis: the structural gene for lysosomal β -galactosidase

Bgl-s - β -galactosidase "systemic", tissue specific activity regulatory gene

Bgl-t - β -galactosidase "temporal", liver specific activity regulatory gene

[Gus] - a genetic locus on chromosome five containing the structural gene and regulatory genes for β -glucuronidase expression

H-7 - a minor histocompatibility locus on chromosome nine

ICM - the inner cell mass (inside cells) of the blastocyst stage embryo

Mod-1 - malic enzyme supernatant

4-Mu - 4-methylumbelliferylone

Trf - transferrin electrophoretic variation

STATEMENT OF THE PROBLEM

The development of metazoans involves the cleavage of the zygote and differentiation of the parts derived by cell division. While genetically hybrid adult metazoans show the expression of both paternally and maternally-derived genetic characters, pregastrular development of most metazoans is notable for the lack of paternal character expression (reviewed by Davidson, 1976). This observation was interpreted as evidence that maternally-derived egg cytoplasmic factors directed the events of early embryogenesis up to the onset of the first morphogenetic differentiations during gastrulation. Strong evidence for this view comes from experiments demonstrating the localization of morphogenetic determinants in egg cytoplasm which restrict the developmental capacity of blastomeres that inherit them (Illmensee and Mahowald, 1974; Curtis, 1962). Eggs containing localized morphogenetic determinants are called "mosaic". Blastomeres isolated from embryos derived from mosaic eggs after the segregation of determinants give rise to partially-developed individuals. Additional evidence comes from "chemical enucleation" experiments performed in sea urchin embryos using the DNA-dependent RNA synthesis inhibitor, actinomycin D. Actinomycin D at dosages which block almost all RNA synthesis in these embryos does not prevent cleavage, but gastrulation is blocked. The protein synthetic rates of treated embryos are nearly normal (Stavy and Gross, 1969). These results indicate that the unfertilized ova of the sea urchin contain large quantities of mRNA that are translated during cleavage. These sequences may contain the information for most pregastrular morphogenesis.

Mouse embryos do not share these characteristics. Mouse embryos are much more sensitive to the effects of DNA-dependent RNA synthesis than are echinoderm eggs. Mouse eggs don't appear to be mosaic. Individual blastomeres isolated after the first or second cleavage division give rise to the complete blastocyst (Tarkowski and Wroblewska, 1967). The blastocyst is a structure with two differentiated cell types. Blastomeres can be isolated from more advanced stages and inserted into blastocyst stage embryos. The isolated blastomere descendants can still contribute to all the structures of the mouse fetus (Gardner, 1968). The expression of paternally-derived characteristics in hybrid mouse embryos occurs during the early cleavage stages (reviewed below) before the first cellular differentiation events are manifest. It is possible that the earliest differentiation of mouse embryo blastomeres is preceded by the differential expression of embryonic genes and not the result of the "precocious" localization of cell fate determinants in the eggs (Gross, 1974).

This work demonstrates that an additional genetic character, expressed before the differentiation of mouse embryo, shows the contribution of the paternal genome. Further, the expression of this character was examined during the first differentiation event in mouse embryogenesis. The events of preimplantation mouse embryogenesis are described in a later section along with additional information on the possible role of gene expression in this process. The next section describes the genetic character examined in this study.

Genetics of Murine Lysosomal β -Galactosidase

The major hydrolytic activity toward the artificial substrates

p-nitrophenyl- β -D-galactoside and 4-methylumbelliferyl- β -D-galactoside at acid pH in neonate and adult mouse tissues is associated with a single enzyme (E.C. 3.2.1.23 β -D-galactoside galactohydrolase; β -galactosidase) coded by a locus, [Bgl], on chromosome nine (Felton, Meisler and Paigen, 1974; Paigen et al., 1976; Breen, Lusi and Paigen, 1977). The structural gene is genetically linked to two regulatory sites. The regulatory sites control enzyme specific activity in mouse tissues during post-partum life. Alleles of the two regulatory phenotypes co-segregate in crosses. Because they cannot be resolved as separate sites either from each other or from the structural gene, they are not accorded the status of separate loci. "[Bgl]" refers to the whole set of regulatory effects and the structural gene and is called a complex.

[Bgl] complex

Structural gene Two electrophoretic patterns of β -galactosidase are found, slow or Bgl-e^b and fast or Bgl-e^a. The electrophoretic difference shows codominant expression in F₁ mice. The difference maps as a single gene on chromosome nine near the H-7 locus (Berger, Breen, Paigen, 1979). The electrophoretic patterns of β -galactosidase from homozygous mice show sub-banding due to charge isomers. The Bgl-e locus affects the migration of all these charge forms simultaneously. There is also an electrophoretic difference between the liver and kidney forms of the enzyme that can be removed by treating the liver form with neuraminidase. But the Bgl-e difference can be seen in the comparison of inbred mouse strains for either kidney or liver electrophoretic patterns. So, the Bgl-e electrophoretic difference probably affects the amino acid portion of the molecule. For these reasons, Bgl-e is

considered to be the site of the structural gene.

Regulation When brain specific activities of β -galactosidase of various inbred mice are examined, they fall into two classes that differ by 1.8 to 2.5-fold (see table 1, page 5). The brain activity level difference between the strains C3H/HeJ (C3H) and DBA/2J (DBA) segregates as a single gene. The phenotype shows additive inheritance in F_1 animals. The locus was mapped to chromosome nine (Felton, Meisler and Paigen, 1974; Meisler, 1976; Berger, Breen, and Paigen, 1979) near the Mod-1 and Trf loci. Liver, heart, and kidney specific activity differences between C3H and DBA are also controlled by the same chromosome nine site and show a similar two-fold variation in activity levels. This site is now called Bgl-s. "S" is for systemic regulator. The activity level difference between mice with the lower activity Bgl-s^d allele (see table 2, page 6) and mice with the higher activity Bgl-s^h allele is caused by a difference in the numbers of enzyme molecules per unit weight of tissue (Meisler, 1976; Paigen et al., 1976). Bgl-s controls enzyme concentration in tissues by controlling rates of enzyme synthesis (Berger, Paigen and Meisler, 1978). The action of Bgl-s is seen from birth to adulthood.

When inbred mouse strains are examined for adult liver specific activity levels, three classes emerge (see table 1). The difference between C3H and DBA mice is explained by the Bgl-s site. The difference between C57BL/6J(B6) and C3H is not explained by the Bgl-s difference, since the brain, heart, and kidney specific activities of these two strains do not differ (Paigen et al., 1976). Further, the Bgl-s difference is expressed from birth, whereas the liver difference between B6

Table 1 β -galactosidase activity level phenotypes

Strain	Relative activity of β -gal. in Brain and Liver ^a		
	<u>Brain</u>	<u>Liver</u>	
		<u>20 days</u>	<u>60 days</u>
C57BL/6J, C57BL/10J	2	2	4
C3H/HeJ, BALB/cJ	2	2	2
DBA/2J, CBA/J	1	1	1

^aThese numbers are the ratios of the tissue specific activities among the inbred strains. Liver specific activities are typically four times higher than brain specific activity levels.

Table 2 [Bgl] haplotypes of strains used in this study

[Bgl] haplotype	Alleles	Strains	Description of phenotype
[Bgl] ^{B6}	e ^b , s ^h , t ^b	C57BL/6J C57BL/10J	e ^b = slow electrophoresis s ^h = high systemic tissue β-gal. spec. activity t ^b = high liver β-gal. at 40-day post-partum
[Bgl] ^C	e ^a , s ^h , t ^b	BALB/cJ C3H/HeHa B10.C ¹	e ^a = fast electrophoresis s ^h = as above ² t ^b = as above
[Bgl] ^{D2}	e ^b , s ^d , t ^d	DBA/2J CBA/J B6.CBA ³ SWR/J C3H/St	e ^b = as above s ^d = low systemic tissue β-gal. spec. activity t ^d = low liver β-gal. at 40-day post-partum

¹ Congenic inbred: BALB/cJ [Bgl]^C haplotype in C57BL/10J.

² BALB/cJ and C3H/HeHa have low liver values relative to C57BL/6J. See text for explanation.

³ Congenic inbred: CBA/J [Bgl]^{D2} haplotype in C57BL/6J.

and C3H is slight until forty days after birth when C3H levels drop away from B6 levels.

B6 and DBA show variation in tissue β -galactosidase levels due to the Bgl-s polymorphism (Paigen *et al.*, 1976). In liver, β -galactosidase activity level differs by two-fold, from birth to forty days, then four-fold thereafter. In the cross of B6 with DBA, the brain specific activity level difference between the two strains segregates as a single gene linked to Mod-1 on chromosome nine with additive expression demonstrating that it is Bgl-s. The four-fold liver activity level difference segregates as a single locus, cosegregating with Bgl-s and showing additive inheritance. Meisler (1976) confirmed the linkage of the liver activity level difference and the brain activity level difference between these two strains and the map location of both to chromosome nine using recombinant inbred strains. Recombinant inbred strains are derived from the F_2 generation from the cross of two inbred strains. They are maintained by brother-sister matings during which new chromosome assortments are fixed in several sub-lines (Bailey, 1971). Additionally, a congenic inbred line was established in which the Bgl-s^d allele was transferred from the CBA/J strain into B6. This congenic strain (B6.CBA) has both the CBA/J low brain and low liver activity levels, indicating that the chromosome nine site alone determines these two differences.

The linkage of the liver activity level difference to the brain activity level difference between DBA/2 (and CBA/J) and B6 is in contrast to the results with C57BL/10Sn (B10) and BALB/cJ. A congenic inbred line, B10.C, was constructed by transferring the H-7 locus of

BALB/cJ into the C57BL/10Sn background. It was found that the Bgl-e^b allele of BALB/cJ was also transferred into the congenic. Breen, Lusi and Paigen (1977) had previously shown that Bgl-s was inseparable from Bgl-e. Paigen *et al.* (1976), as discussed above, found one genetic site for the liver activity level difference between DBA/2J (and CBA/J) and B6 that cosegregated in crosses with Bgl-s and co-transferred with Bgl-s in the construction of a congenic inbred line. So, it was surprising when the B10.C congenic had a liver specific activity developmental pattern like B10. This suggested that sites unlinked to [Bgl] might also participate in the liver activity level determination.

Berger, Breen and Paigen (1979) used recombinant inbred lines from the progenitors C57BL/6ByJ (B6) and BALB/cByJ (BALB) to determine linkage between Bgl-e and the sites for the liver activity determination difference in B6 and BALB. Among seven recombinant inbred lines, two showed recombination between Bgl-e and the liver activity determinants. The independent segregation of these sites was confirmed by these workers in a cross between B6 and BALB which showed no correlation between Bgl-e type and liver activity levels in F₂ or backcross progeny.

Liver activity levels for β -galactosidase are determined both by a site that cosegregates with Bgl-s and by sites that assort independently of Bgl-e. The site linked to Bgl-s is called Bgl-t. Bgl-t and the unlinked liver activity determining sites regulate liver activity by controlling rates of enzyme synthesis (Berger, Paigen and Meisler, 1978). The liver phenotype, determined by Bgl-t and the unlinked site, is called the temporal effect and these sites collectively are referred to as temporal genes. Bgl-s acts in cis-chromosomal fashion on Bgl-e

expression (Berger and Paigen, 1979). It is not known if Bgl-t acts in this way.

[Bgl] haplotypes Alleles for Bgl-e s t exist in only three combinations in inbred mouse strains. The three combinations and mouse strains with each combination are presented in table 2 (see page 6). Each combination is called a haplotype and is designated by a type strain with that haplotype.

The existence of congenic strains of mice with different haplotypes of the [Bgl] complex on the C57BL background provided the opportunity to learn more about the regulatory elements of the complex by comparing their effect on β -galactosidase expression at a different time in the life of the mouse. My particular interest was the preimplantation period of mouse embryogenesis.

The locus [Gus], on chromosome five, codes for an enzyme (β -glucuronidase; E.C. 3.2.1 31) that is the major hydrolytic activity toward the artificial substrates p-nitrophenyl- β -D-glucuronide and 4-methylumbelliferyl- β -D-glucuronide at acid pH. β -Glucuronidase is found in the microsomes and lysosomes, while β -galactosidase is located in the lysosomes (Ganschow and Paigen, 1967). The two activities show coordinate expression in many tissues, part of which may be due to similarities in the types of regulatory elements associated with each genetic locus (Meisler and Paigen, 1972). The chromosome five complex has the structural gene and contains regulatory determinants much like those in [Bgl]. The structural gene in the [Gus] complex is expressed in preimplantation mouse embryos (see below). It seemed possible that the [Bgl] complex structural gene might be expressed at

this time as well.

Two questions could be asked about gene expression during mouse development by looking at [Bgl] complex expression in preimplantation mouse embryos. The first is the general question about genetic control of preimplantation development. To what extent do maternally-inherited cytoplasmic factors contribute to developmental expression and to what extent does transcription of embryonic genes contribute to developmental expression? In this work, the question is addressed by an examination of whether the maternal genotype or embryonic genotype at the [Bgl] complex determines the pattern of β -galactosidase expression in preimplantation embryos. In part, this is a question of timing, since ultimately the embryonic genes are activated and the progeny genotype does determine the pattern of structural gene expression by parturition.

The second question is, what facets of preimplantation embryonic β -galactosidase expression are determined by the [Bgl] genetic complex? This question has two parts. First, do different [Bgl] haplotypes affect the timing for the initiation of transcription? This would imply that sites linked to the structural gene not only regulate rates of synthesis, but also act as elements of a developmental clock-like mechanism for gene activation. Second, do regulatory sites like the liver specific Bgl-t or its distal modifiers express themselves in either of the two cell types elaborated in the preimplantation mouse embryo? This requires an interstrain comparison of the two cell types for β -galactosidase activity levels.

The first question requires the β -galactosidase activity expressed

in embryos to be the product of [Bgl] complex. The most direct and conclusive way to test this would be to examine the electrophoretic properties of the enzyme in homozygous and heterozygous embryos. This was attempted, but with no success. However, the structural gene product rates of synthesis are controlled by regulatory sites that map in close proximity to the structural gene. I did not measure rates of enzyme synthesis. But between the congenic inbred strain B6.CBA, with low rates of synthesis alleles of the [Bgl] complex and the B6 strain, with high rates of synthesis alleles, the relative activity levels should be determined only by the relative difference of the rates of enzyme synthesis specified by differences at [Bgl]. Because the actions of the [Bgl] regulatory sites are on the synthesis of the Bgl-e gene product in adult tissues, a demonstration of the action of [Bgl] regulatory sites on β -galactosidase enzyme activity levels in embryos would by analogy suggest that the structural gene in the [Bgl] complex is the source of the enzyme. Once it is established that the [Bgl] complex regulates the expression of the embryonic activity levels, then the timing of the putative structural gene transcription can be determined by comparing embryos heterozygous for the regulatory alleles of [Bgl] with groups of embryos homozygous for the regulatory alleles of [Bgl] for differences in their mean activity levels of β -galactosidase.

If the answer to the first question is in favor of transcription of the [Bgl] locus, then the second question about [Bgl] complex regulatory element influences on β -galactosidase expression can be asked.

Other Considerations in the Analysis of Strain Variation for β -Galactosidase

The issues raised above are addressed in an analysis of the sources of quantitative variation between the embryos of different inbred strains for β -galactosidase activity levels. For this type of analysis, it is necessary to examine strains for different rates of development which could result in differences in β -galactosidase levels, but not act through the [Bg1] complex. Cleavage rate variations of embryos between inbred strains were examined as an index of the relative developmental rate differences between strains. Since major changes in embryonic β -galactosidase activity levels occur during the elaboration of two cell types, these two cell types were separated and each assayed for activity. The total embryonic cleavage rate data might not be an adequate estimate of developmental rate variation if β -galactosidase activity expression was the exclusive domain of either cell type and each strain had a different timing for the elaboration of the two cell types.

Preimplantation Mouse Embryogenesis

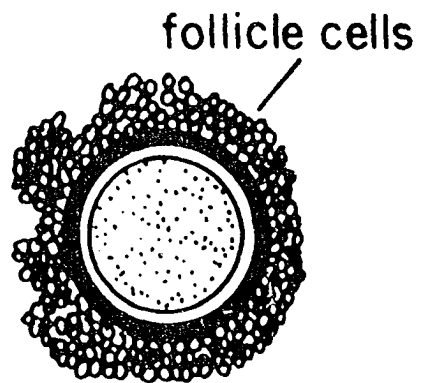
The study of cellular differentiation in situ and of the role of gene expression in the process is a difficult undertaking. Yet there is some appeal to studying a natural developmental change and differentiation event. The preimplantation mouse embryo is transiently free living. It can be removed from the oviduct, cultured for some time and returned to the oviduct. Embryos handled in this manner are still capable of normal development to adulthood. During the preimplantation stage, one cell type (the zygote) differentiates into what appears to be two

morphologically distinct cell types. Preimplantation stage mouse embryogenesis lasts about one hundred hours. Net growth of the embryo does not occur. The first cleavages are marked by a decrease in cytoplasmic volume, total dry weight and protein (Biggers and Borland, 1976; Brinster, 1967; Abramczuk and Sawicki, 1974). During this period, a series of morphological events occurs that can be correlated with specific cell numbers (see fig. 1, page 14). The events include compaction of the blastomeres at the eight-cell stage, the generation of intracellular fluid-filled cavities at the twenty to thirty-cell stage with culmination in a large intercellular fluid-filled cavity, the blastocoele, at the thirty to forty-cell stage (Ducibella et al., 1977; Smith and McLaren, 1977). The production of the blastocoele is accompanied by the establishment of the two distinct cell types, the trophectoderm and the inner cell mass. Each cell type is destined to a separate fate in the peri- and post-implantation stage embryo. Trophectoderm will contribute to the extraembryonic membranes while the inner cell mass (ICM) will contribute to the embryo proper (Gardner and Papaioannou, 1975; Rossant and Papaioannou, 1977).

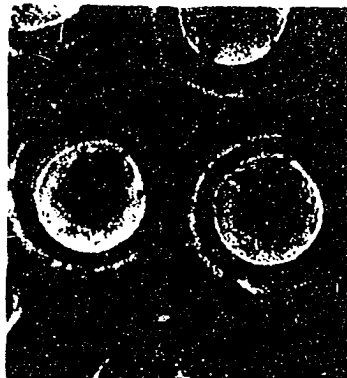
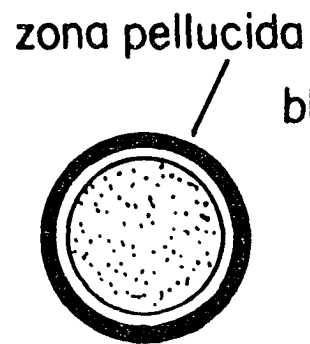
Developmental changes occur at two levels, subcellular and cellular. At the subcellular level, the earliest cleavages are marked by a shift in the carbon sources the embryo can utilize for energy (Biggers and Borland, 1976). Between the two and four-cell stages, nucleoli differentiate from a primary, non-granular form to a mature form (Hillman and Tasca, 1969). The synthesis of ribosomal RNA begins at the late two-cell to early four-cell stage (Woodland and Graham, 1969). Mobilization of ribosomes for protein synthesis is noticeable

Figure 1. Depiction of preimplantation mouse embryogenesis [The photographs are of embryos of the random bred line Ha/ICR taken at the indicated number of hours after artificial insemination (A.I.). The drawings are schematic representations of the photographs with the major structures labeled].

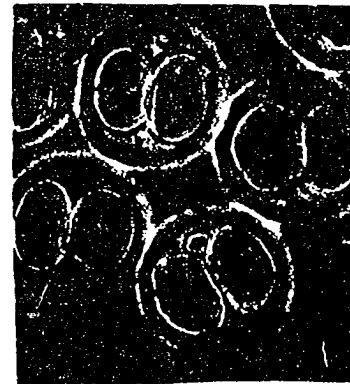
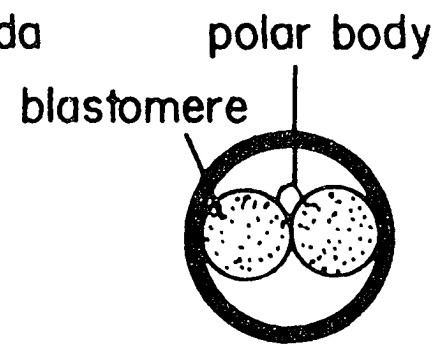
unfertilized egg
14 hrs. post-HCG



fertilized egg
10 hrs. post-A.I.



2-cell stage
23 hrs. post-A.I.



4-cell stage
47 hrs. post-A.I.

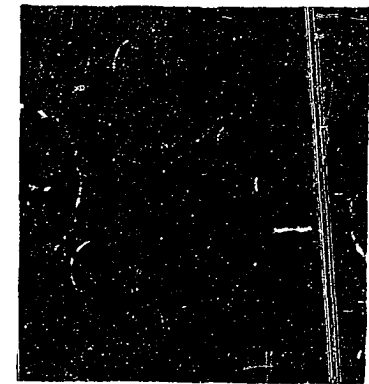
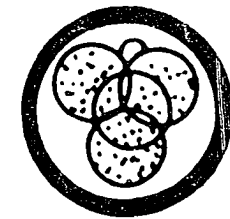
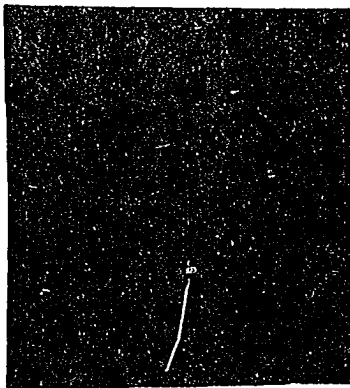
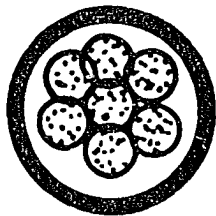


Figure 1. (continued)

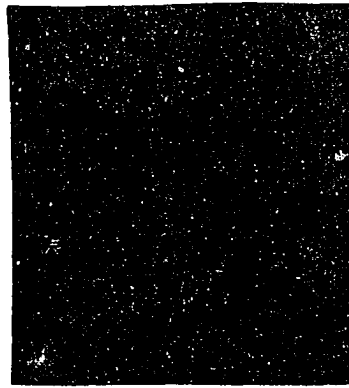
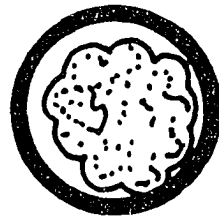
8-cell stage

60hrs. post-A.I.



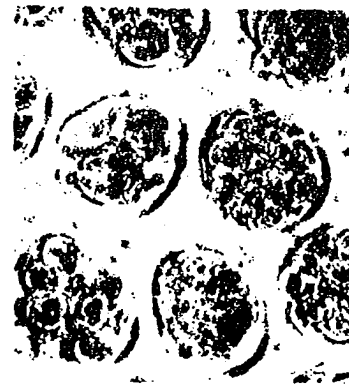
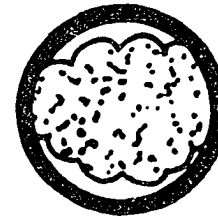
early morula stage
(compacted)

66 hrs. post-
A.I.
14 cells



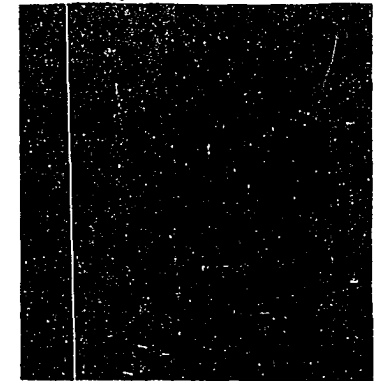
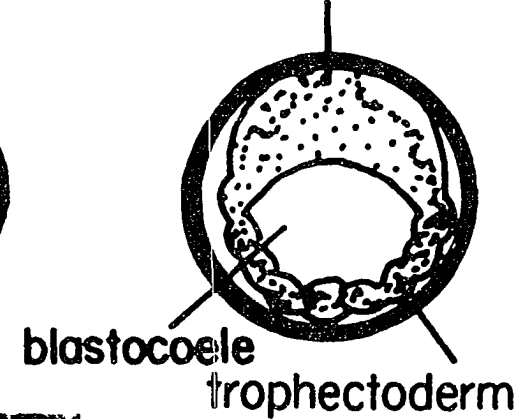
morula stage

72 hrs. post-
A.I.
20 cells



blastocyst

84 hrs. post-
A.I.
50 cells
inside cells



by the morula stage. Protein synthesis rates increase at this time. Additionally, there are changes in many enzyme activity levels and in the antigenicity of many cell surface components (Willison and Stern, 1978; Solter and Knowles, 1978; reviewed in Epstein, 1975). Two-dimensional gel electrophoresis of newly-synthesized proteins shows that at each cleavage there is a change in the protein synthetic pattern (Levinson et al., 1978).

Underlying the processes of compaction and cavitation (formation of the blastocoele) are the organization of microvilli, intercellular junctions (Ducibella et al., 1975; Ducibella et al., 1977; Nadijcka and Hillman, 1974) and intercellular differentiation. By the time the ICM and trophectoderm have segregated, they are synthesizing different arrays of proteins (Van Blerkom, Barton and Johnson, 1976) and they have acquired several different cellular properties (Barlow, Owen and Graham, 1972; Gardner, 1972). Electronphotomicrographs show that the most striking differences in subcellular organization between the ICM and trophectoderm are the type of intercellular contacts these cells establish (Nadijcka and Hillman, 1974). While the differentiation itself involves the generation of only two morphological cell types by the time of implantation, the process occurs against a backdrop of complex developmental changes. An intriguing possibility is that the differentiation of ICM and trophectoderm involves post-fertilization gene transcription and so is analogous to later differentiation events. There is some evidence that gene transcription in early embryos does contribute to preimplantation developmental changes. Already, studies with inhibitors of DNA-dependent RNA synthesis suggest that RNA synthesis

is necessary for normal preimplantation development in the mouse (reviewed below). Further, by looking for expression of paternally-derived variants of glucose phosphate isomerase and β -glucuronidase during the cleavage stage, Brinster (1973), Chapman, Whitten and Ruddle (1971) and Wudl and Chapman (1976) found evidence for the transcription of the structural genes of these enzymes by the eight-cell stage. Paternally-derived minor histocompatibility alloantigens were detected on eight-cell mouse embryos by Muggleton-Harris and Johnson (1976). Since these experiments depended on detection of the active enzyme or membrane antigen, they are evidence for nuclear genome expression in preimplantation (reviewed below). The actual contribution of any of these molecules to the functions of the cleavage stage embryo is an unsettled question. But these examples do open the possibility that nuclear genes have a part in directing some features of early development.

The Connection Between Cleavage Stage RNA Synthesis, Morphogenesis and Differentiation

There is no clear DNA-dependent RNA synthesis in one-cell mouse embryos. [^3H]-Guanosine, [^3H]-adenosine and [^3H]-uridine are taken up by one-cell mouse embryos with less than 10% of the uridine, 5% of the guanosine, and 1% of the adenosine counts incorporated in TCA insoluble material (Young, Sweeney and Bedford, 1978; Young and Sweeney, 1979). Immediately after fertilization, the bulk of labeled uridine nucleotides are UMP, UDP and uridine diphosphate glucose with UTP representing only 3% of the total (Young, Sweeney and Bedford, 1978). Mid-to-late one-cell embryos have 50% of their uridine nucleotides as

UTP (Clegg and Piko, 1977). Young, Sweeney and Bedford (1978) do not present data on the percent [^3H]-uridine in the soluble fraction, so the actual amount of UTP available for RNA synthesis can't be determined. Clegg and Piko's data show that counts in UTP are 30% of the soluble total by the late one-cell stage. Of the acid-insoluble counts, only 30%, 70% and 3% are RNAase sensitive for uridine, adenosine and guanosine, respectively (Young, Sweeney and Bedford, 1978; Clegg and Piko, 1977). The guanosine incorporation has been characterized as capping of RNA (Young, 1977). The RNAase sensitive adenosine incorporation is cytoplasmic adenylation of RNA. This adenylation is stimulated rather than inhibited by actinomycin D at levels which inhibit 50% of one-cell embryos from cleaving (Young and Sweeney, 1979). Much of the incorporation was characterized as 3'-adenylation of RNA (4S) and turnover of CCA segments in tRNA. This activity is a carryover of processes occurring in unfertilized eggs. Some of the incorporated uridine was found to be in internal positions of RNA suggesting a very low level of RNA synthesis (Young, Sweeney, and Bedford, 1978). Clegg and Piko (1977) estimated that the rate of RNA synthesis based on uridine incorporation was 2×10^{-3} picomoles per embryo. This figure was arrived at after correction for the fact that only 35% of the acid-insoluble counts in late one-cell embryos are alkali sensitive. The intracellular localization of the incorporated material was not determined.

The first cleavage is slightly sensitive to actinomycin D and α -amanitin (Golbus, Calarco and Epstein, 1973). In vitro, the levels of α -amanitin used by Golbus, Calarco and Epstein are supposed to inhibit

only the form II RNA polymerase (form II synthesizes HnRNA, Chambon, 1975). No single class of RNA synthesis is identifiable at this stage. Versteegh, Hearn, and Warner (1975) could not detect an RNA polymerase II activity in the one-cell embryo. So, the sensitivity of the first cleavage is puzzling indeed. The first two cleavages don't appear to require mitochondrial gene activity (Piko and Chase, 1973). However, it has been suggested that actinomycin D and α -amanitin may affect cellular respiration. Golbus, Calarco and Epstein (1973) took the precaution of assaying ATP levels in control, actinomycin D and α -amanitin treated one-cell embryos. They found no statistically significant difference between the ATP levels of the three groups after 24 hours. A most interesting observation is that of Warner and Hearn (1977a), who found that α -amanitin slightly suppressed the degree that interconverted [^3H]-deoxycytidine (from [^3H]-uridine) was incorporated into a high molecular weight, DNAase sensitive material. They suggested that DNA polymerase messenger might be affected by the drug or that synthesis of primer RNA for DNA replication was inhibited. To date, there are no direct studies on the short-term effects of α -amanitin or actinomycin D on DNA synthesis in mouse embryos, nor are there any studies at the early stages on sensitivity of protein synthesis to α -amanitin. If the drug sensitivity data are really an indication of a necessary mRNA synthesis, possible candidates are histone synthesis, DNA polymerase, actin and tubulin (Warner and Hearn, 1977a; Schultz, Letourneau and Wassarman, 1979). Among the earliest mRNA synthesis in sea urchin embryogenesis is transcription of histone sequences (Gross et al., 1973). Further, either histone synthesis or DNA polymerase

synthesis might account for depression of DNA synthesis. Presently, one cannot assert that the inhibitory effects are due to the suppression of vital messenger RNA synthesis. Such an interpretation, although tempting, is based largely on the known primary in vitro effects of α -amanitin and ignorance of the total in vivo effects at this stage.

Distinct species of RNA are synthesized at the two-cell stage. Shifts in the phosphorylation of [3 H]-uridine, already occurring in late one-cell embryos result in most of the precursor being converted to UTP by the two-cell stage (Clegg and Piko, 1977; Daentl and Epstein, 1971). This change precedes the first detectable RNA synthesis in the embryo.

Ribosomal RNA synthesis Three lines of evidence suggest that ribosomal RNA synthesis is beginning in late two-cell and early four-cell embryos. First, electronphotomicrographs show that the granular portion of the nucleolar organizer is being elaborated at this time. [3 H]-uridine labeled acid-insoluble material first appears in the developing granulosa (Hillman and Tasca, 1969). Engel, Zenzes and Schmid (1977) used a Ag-As staining technique to determine when active nucleolar organizers first appear. The Ag-As technique has been shown to stain only active organizers. At the late two-cell stage, only one chromosome of the six (three pairs of homologous chromosomes) that will ultimately be active was stained. By the four-cell stage, four-to-six chromosomes were stainable. In the eight-cell embryo, all six chromosomes were stainable. Banding of metaphase-spread chromosome preparations confirmed that the staining occurred at specific locales on the three pairs of chromosomes previously identified by Henderson et al. (1974)

to contain the rRNA loci. Thirdly, some [^3H]-uridine is incorporated into RNA that sediments like rRNA and runs on acrylamide gels like rRNA (Woodland and Graham, 1969; Knowland and Graham, 1972; Clegg and Piko, 1975). However, for the two-cell stage, the data are not definitive because of large background of heterodisperse RNA that interferes with rRNA detection. At the four-cell stage, the peaks of incorporation into 18S and 28S RNA are very clear.

The onset of low molecular weight RNA synthesis RNA in the 4S to 5S range is synthesized beginning at the four-to-eight-cell stage (Woodland and Graham, 1969). The exact nature of material sedimenting in this range is not clear for the four-cell stage. Woodland and Graham reported that the labeled 4S RNA from eight-cell embryos contained large amounts of pseudouricil. These two characteristics suggest that it is transfer RNA. [^3H]-Uridine used to label this material did not show significant interconversion to cytosine, so the counts in the 4S RNA represented synthesis and not turnover of CCA terminal nucleotides. Knowland and Graham (1972) present acrylamide gels of [^3H]-uridine labeled RNA from two-cell embryos which show a low molecular component that could be 4S RNA or 5S RNA.

The onset of messenger RNA synthesis The problem with direct detection of mRNA synthesis stems from its heterodisperse size and its low level of synthesis relative to other forms. On the other hand, most messages are polyadenylated, permitting tentative identification on this basis. This approach has been used by two groups (Levey, Stull and Brinster, 1978; Warner and Hearn, 1977b).

Levey, Stull and Brinster (1978) found that 6.7% of the [^3H]-

uridine counts in labeled RNA from two-cell embryos bound to an oligo (dT) cellulose column. Gel electrophoresis of the polyadenylated RNA in acrylamide showed it to be heterodisperse, spanning 5S to 28S. The 3' poly(A) tails were estimated to be 85 to 100 nucleotides long. Warner and Hearn (1977b) reported lower percentages of polyadenylated RNA than Levey, Stull and Brinster. The size distribution profile was also different. The two groups used different techniques for the isolation of RNA and the estimation of the percent material in poly(A). Warner and Hearn's data show the polyadenylated RNA synthesis to be more resistant to actinomycin D in the range of 0.01 to 1 $\mu\text{g/ml}$ than bulk RNA. Nuclear RNA accounts for only five percent of the total cellular incorporation, so it is not likely that synthesis and turnover of nuclear RNA accounts for the findings (Levey and Brinster, 1978).

The poly(A) RNA synthesis detected by Levey and Brinster (1978) is particularly sensitive to the inhibitor α -amanitin, both with respect to time for total inhibition and dosage. An α -amanitin concentration of 1-10 $\mu\text{g/ml}$ stopped labeling of polyadenylated RNA within thirty minutes to one hour. In contrast, total RNA synthesis is inhibited only ten to fifteen percent in the same amount of time at these dosages.

Another line of evidence for mRNA synthesis during preimplantation is based on changes in the total sequence complexity of RNA from single copy DNA sequences. Church and Schultz (1974) report a slight increase in complexity occurring from the eight-to-sixteen-cell stage to the blastocyst stage.

Summary Detection of all major classes of RNA synthesis is

possible at the four-cell stage. However, Moore (1975) raises the possibility that some synthesis in two-cell embryos is in the polar body and not in the embryo. When rates of RNA synthesis are measured after the four-cell stage, these are by-an-large estimates of ribosomal RNA (Piko, 1970; Ellem and Gwatkin, 1968). Also, the estimates of total RNA content are largely of ribosomal RNA and possibly accumulated 4S and 5S RNA. Olds, Stern and Biggers (1973) reported that total RNA content of the embryo increases three-fold during cleavage, starting at the eight-cell stage. The RNA synthesis rate data of Clegg and Piko show two-fold increases in rates between the first and second cleavage then, four-fold and eight-fold increases occurring at the third cleavage and at cavitation. These data predict increases in total RNA content that agree favorably with the actual measurements (Clegg and Piko, 1977; Daentl and Epstein, 1971; Olds, Stern and Biggers, 1973; Young, Stull and Brinster, 1973). Polyadenylated RNA remains at about two to three percent of the total incorporated counts from the eight-cell stage to the blastocyst stage (Levey, Stull and Brinster, 1978; Warner and Hearn, 1977b). Levey, Stull and Brinster do report a larger percentage for two-cell embryos. Moore (1975) found that two-cell mouse embryos showed a distinct nucleoplasmic labeling, but no nucleolar label with [^3H]-UTP.

The Relationship of Early RNA Synthesis to Protein Synthesis and Cleavage

There are three lines of evidence that the RNA synthesis detected in cleavage stage mouse embryos is immediately important to cleavage. First, the estimates above show that ribosomal RNA synthesis

contributes significantly to the mass of the ribosomes. Part of the RNA synthesized does include polyadenylated RNA. Both facts increase in importance when the role of ongoing and increasing protein synthesis is accessed. Secondly, α -amanitin, actinomycin D, bromodeoxyuridine and cordycepin affect cleavages and cavitation. Thirdly, genetic evidence from the mouse shows clear examples of paternal gene expression by the eight-cell stage.

Cleavage and protein synthesis

Protein synthesis is required at all cell stages for cleavage and differentiation of the mouse preimplantation embryo (Monesi et al., 1970, Hillman and Tasca, 1969; Thompson and Biggers, 1966).

Actinomycin D effects

Actinomycin D was the inhibitor used in the earliest mouse embryo work. Actinomycin D in the range of 0.01-0.04 $\mu\text{g/ml}$ does stop rRNA synthesis (Piko, 1970; Zybier and Penman, 1971). Ellem and Gwatkin (1968) identified an actinomycin D resistant DNA-like RNA synthesis in four-cell embryos. Warner and Hearn (1977b) found polyadenylated RNA synthesis from eight-cell and blastocyst stage embryo to be more resistant to 0.01 $\mu\text{g/ml}$ actinomycin D than total RNA synthesis, but total counts into poly(A) RNA were probably depressed. The same dosage of actinomycin D is effective in reducing the rate of [^3H]-leucine incorporated into protein in two-cell embryos by sixty percent, but is not so potent at later stages (Monesi et al., 1970). Still, continuous exposure to the inhibitor results in a twenty to thirty percent reduction in incorporation at later stages. Cleavages show a similar sensitivity. A two-cell embryo is extremely sensitive, while four and

eight-cell stage embryos are less so. The data of Mollinaro, Siracusa and Monesi (1972) suggest a cell cycle variation in the effects of actinomycin D on cleavage for the two-cell embryo.

Actinomycin D does bind to DNA and its most immediate effect is to inhibit DNA-dependent RNA synthesis (Hurwitz et al., 1962; Reich, Goldberg and Rabinovitz, 1962). At the lower dosages used in mouse embryo work (0.01-1 $\mu\text{g/ml}$), the ribosomal RNA synthesis should be most affected (Zybler and Penman, 1971; Epstein, 1975; Warner and Hearn, 1977b; Ellem and Gwatkin, 1968; Piko, 1970). Low molecular weight RNA synthesis is more resistant but also is suppressed (Ellem and Gwatkin, 1968; Piko, 1970). Quantitatively, the contribution of ribosomal RNA synthesis to the ribosome pool is negligible prior to the eight-cell stage. Actinomycin D is also reported to have additional effects on cells not related to the suppression of RNA synthesis. First, it does affect DNA polymerase activity at levels of 2-10 $\mu\text{g/ml}$ in vitro (Hurwitz et al., 1962). Honig and Rabinovitz (1965) reported a peculiar glucose reversible inhibition of protein synthesis in sarcoma cells. Laszlo et al. (1966) found respiration was slightly depressed in mammalian cells at lower dosages. Pasten and Friedman, (1968) showed a depression of phospholipid synthesis, not totally accounted for by the depression of protein synthesis, occurred in chick fibroblast cells at levels of 0.5 $\mu\text{g/ml}$.

Of interest are reports that poly(adenosine diphosphate ribose) polymerase activity is affected by actinomycin D (Claycomb, 1976; Yoshihara, 1972). The literature has conflicting reports of this

sensitivity and its direction. Poly(ADP ribose) seems to have some role in histone phosphorylation, DNA replication and NAD concentration in cells. Conversion of [^3H]-adenosine to [^3H]poly(ADP ribose) does occur in one-cell mouse embryos (Young and Sweeney, 1979).

So, there is considerable reason to be skeptical that the only or most immediate effect of actinomycin D is mediated through its suppression of RNA synthesis. It may be informative to see if the cell cycle related inhibition of the second cleavage reported by Molinaro, Siracusa and Monesi (1972) is related to suppression of a particular set of proteins. Also, studies of histone deposition or phosphorylation, or DNA synthesis in the presence of actinomycin D might shed light on actinomycin D effects in the first three cleavages. At this point, there is no clear indication that the coincidence in dosages that suppress cleavage and protein synthesis are related to suppression of RNA synthesis.

α -Amanitin effects

α -Amanitin, like actinomycin D, is a potent inhibitor of cleavages and cavitation (Golbus, Calarco and Epstein, 1973; Levey, Troike and Brinster, 1977; Warner and Versteegh, 1974). At low dosages (1 $\mu\text{g}/\text{ml}$) its primary effect is suppression of the form II RNA polymerase (Chambon, 1975). Low dosages of α -amanitin are reported to disrupt nucleolar structure in mouse embryos (Golbus, Calarco and Epstein, 1973) and mouse liver (Marinozzi and Flume, 1971). Ribosomal RNA synthesis is also suppressed at moderate dosages of α -amanitin (Levey and Brinster, 1978; Warner and Hearn, 1977a). The effect seems to be on synthesis and

maturation of the 45S rRNA precursor (Levey and Brinster, 1978). But, the inhibition of rRNA synthesis at low dosages is far from complete. Polyadenylated RNA synthesis is extremely susceptible. So, I will confine the discussion of α -amanitin effects on morphogenesis to cases where the dose was 11 μ g/ml or less.

The first cleavage is marginally sensitive to α -amanitin. Golbus, Calarco and Epstein (1973) found fifty percent inhibition while Levey, Troike and Brinster (1977) found about twenty-five percent inhibition. The second cleavage is also slightly inhibited when two-cell embryos are incubated in α -amanitin. Incubation of morulas in α -amanitin prevents ten to thirty percent from cavitating (Levey, Troike and Brinster, 1977). The inhibitory effects of α -amanitin are actually latent. So, although one cleavage is possible, successive cleavages are severely repressed. Embryos cultured continuously in α -amanitin from the one-cell and two-cell stages don't achieve the morula stage. Eight-cell stage embryos do not cleave to the blastocyst stage in the presence of α -amanitin (Levey, Troike and Brinster, 1977; Golbus, Calarco and Epstein, 1973; Warner and Versteegh, 1974). Protein synthesis rates are reduced 85% to 95% in morula continuously exposed from the one-cell and two-cell stages (Golbus, Calarco and Epstein, 1973). Several investigators report that the specific polypeptide synthesis changes that occur at cavitation are altered by exposure of the embryo to α -amanitin. Golbus, Calarco and Epstein (1973) showed that the HPRT activity increase in morula stage embryos was prevented if embryos were incubated with α -amanitin from the two-cell stage, but not from the four-cell stage. This information does suggest

that continuous synthesis of messenger RNA is required for maintenance of protein synthesis, cleavage and cavitation. The data also suggest delayed utilization of mRNA during cleavage.

Cordycepin and 5-bromodeoxyuridine effects

Cordycepin is an adenosine analog that does inhibit polyadenylation of RNA (Adesnick et al., 1972). Exposure of mouse cleavage stage embryos to 25 to 50 ug/ml of cordycepin inhibits the transition to blastocyst (Levey and Brinster, 1978). These same dosages reduce [³H]-uridine incorporation into RNA. While polyadenylated RNA is affected most completely, ribosomal RNA is significantly reduced as well. [³H]-Leucine incorporation is only slightly affected over the short term, but data for long-term suppression were not presented. The lack of any effect of cordycepin on the second cleavage, coupled with the suppression of rRNA synthesis raises some doubt about the primary effect of the inhibitor.

BrdU is incorporated into DNA in the place of thymidine (Pollard, Baran and Bachvarova, 1976). BrdU-substituted DNA has an altered affinity for proteins. This could alter the pattern of transcription. Continuous culture of embryos to BrdU at 1×10^{-5} M does prevent cavitation (Golbus and Epstein, 1974; Pollard, Baran and Bachvarova, 1976). The effects of BrdU are uncertain. Garner (1974) reported that BrdU-treated embryos produced blastocyst with fewer numbers than controls. BrdU has been shown to suppress the activity of ribonucleotide reductase causing deoxycytidine starvation (Meuth and Green, 1974). The inhibition of blastocyst formation by BrdU is partially reversible by deoxycytidine (Pollard, Baran and Bachvarova, 1976).

Summary

Not all of the inhibitory effects of RNA synthesis inhibitors on cleavage stage embryos are explainable by their known primary in vitro effects. The best evidence for a role of RNA synthesis during cleavage comes from the use of α -amanitin at low doses. The complete and quick suppression of polyadenylated RNA synthesis with moderate suppression of other RNA species suggest an effect on cleavage mediated by polyadenylated RNA. The patterns of peptides synthesized by the cleavage stage mouse embryo are constantly changing (Levinson et al., 1978; Handyside and Johnson, 1978). The rates of protein synthesis increase largely after the eight-cell stage (Epstein and Smith, 1975). So, new messenger RNA synthesis could be required to maintain ongoing synthesis of some peptides or initiate new protein synthesis patterns. Still, at early cleavage stages, one can't resolve the effects of α -amanitin. α -Amanitin could be just reducing overall protein synthesis or it could be selectively disrupting a vital function. α -Amanitin does disrupt rRNA synthesis. While rRNA synthesis is not quantitatively large before the eight-cell stage, the transport of new ribosome subunits to the cytoplasm may be important for maintenance of protein synthesis (Tata, 1968). Vital protein synthesis suggested by the literature includes rRNA processing proteins (Levey and Brinster, 1978; Warner and Hearn, 1977b), histones (Gross et al., 1973), actin and tubulin (Abreu and Brinster, 1978), DNA polymerase (Warner and Hearn, 1977b), cell surface antigens (Kemler et al., 1977).

Finally, the effects of inhibitors on differentiation or determination are not well-defined. Numerous reports show that trophectoderm outgrowths are relatively resistant to the inhibitors mentioned above (Glass, Spindle and Pederson, 1976; Pederson and Spindle, 1977; Rowinski, Solter and Koprowski, 1975). ICM derivatives are sensitive. But, by this point, the trophectoderm is differentiated and for many reasons appears to be committed to its fate. Data from studies on late morula suggest α -amanitin does have very specific effects on protein synthesis (Braude, 1979). But whether the critical peptides that affect cavitation are tissue specific or not isn't known.

Genetic Evidence for a Connection Between Early Messenger-like RNA Synthesis and Preimplantation Events

The expression of paternally-derived genetic traits in hybrids is a long-used method for estimating the timing of gene expression (Davidson, 1976). The time of death in individuals presumably homozygous for recessive lethal genes is another means. The action of lethal genes in preimplantation mouse embryos points to possible required transcription.

Alleles of several genetic loci act as recessive lethals during preimplantation. These are alleles of the T-locus on chromosome seventeen (t^{12} , t^{w32}), alleles of the agouti locus (a^X , A^Y), oligosyndactylism (OS) and Tail-Short (TS).

The literature on embryonic lethals is reviewed in several articles (Bennett, 1975; Sherman and Wudl, 1977; Wudl, Sherman and Hillman, 1977; McLaren, 1976; Chapman, West and Adler, 1977).

Sherman and Wudl provide an excellent analysis of the T-complex.

McLaren provides insight into the lethal pseudoalleles of the agouti complex and their interaction with the genetic background.

T-complex

Embryos homozygous for the t^{12} or t^{w32} alleles die at the early-to-late morula stage. Heterozygous embryos survive to adulthood. There is no difference in the viability of heterozygous embryos when the wild-type allele is introduced via the sperm or egg. Analysis of the specific defects has not been fruitful in providing a molecular explanation for the embryonic arrest and death. The research field has become divided in the last few years between workers who propose that a breakdown in very specific cell-cell interaction due to defects in the temporal or spatial expression of cell surface antigens causes the lethality (Artzt et al., 1973; Artzt and Bennett, 1975; Artzt, Bennett and Jacob, 1974; Bennett, 1975; Kemler et al., 1977) and those who suggest that the biochemical lesion is not at the cell surface nor specific to any cell type (Wudl and Sherman, 1976; Wudl, Sherman, and Hillman, 1977). The fact that the t^{12} and t^{w32} mutations do cause death of the embryo at the morula stage does suggest that some gene product made by the T-locus must be present in the cleavage stage embryo for successful cleavage to the blastocyst stage.

Agouti locus

The agouti yellow allele of the agouti locus is a recessive lethal. In the heterozygous state, it is a dominant gene for yellow coat color and is associated with obesity and other physiological defects (Heston and Vlahakis, 1968). The timing of embryonic death

depends on the mouse strain in which the mutation is tested (McLaren, 1976). Abnormalities can be detected as early as the morula stage in culture.

There is no reason to believe that yellow pigment production, obesity or other problems caused by the A^Y allele are associated with the death of homozygous embryos. Dickies (1962) found an allele of the agouti locus which causes yellow hair pigmentation, obesity, etc., but is not lethal in the homozygous state. Here, as in the case with the T-locus, the lethality of A^Y allele suggest that the genetic constitution of the embryo is important in development as early as the morula stage. But, the basis of the defect is not known (Calarco and Pederson, 1976; Cizadlo and Granholm, 1978).

Single gene variation and the timing of paternal gene expression

Glucose-phosphate isomerase (GPI) is a dimeric enzyme with two electrophoretic alleles among inbred mice (Carter and Parr, 1967). The alleles can be easily separated on starch gels. Heterozygotes show both parental alleles and a heteropolymeric band that migrates in an intermediate position. The heteropolymeric and maternal bands were detected in hybrid blastocysts by Chapman, Whitten and Ruddle (1971). Brinster (1973) used a larger sample size to detect paternally-derived enzyme at the eight-cell stage.

β -Glucuronidase activity levels increase about one-hundred fold between the two-cell stage and the blastocyst stage (Wudl and Chapman, 1976). A single genetic complex on chromosome five has sites for regulation of enzyme levels and codes for the structural gene. Variants exist for the enzyme activity levels of preimplantation embryos

and the heat stability of the embryonic enzyme. The F_1 ($\underline{\text{Gus}}^h$, low activity, heat-labile phenotype $\text{♀} \times \underline{\text{Gus}}^b$, high activity, heat-resistant ♂) has higher activity values than homozygous maternal-type embryos at the two-cell stage. The F_1 activity difference continues to increase throughout preimplantation. When embryo homogenates were heat treated, F_1 embryo activity inactivated as if it had both labile and resistant enzyme. In the previous experiment (Wudl and Chapman, 1976), the $\underline{\text{Gus}}^h$ allele was on the C3H/HeHa background, while the $\underline{\text{Gus}}^b$ allele was on the B6 background. This left the question of whether the differences in activity level were due to the $[\underline{\text{Gus}}]$ complex or due to other differences in the genetic background. Chapman *et al.* (1976) examined C57BL/6- $\underline{\text{Gus}}^h$ embryonic activity levels of β -glucuronidase along with the embryos from a mating of the congenic inbred mouse, C57BL/6- $\underline{\text{Gus}}^h$, and C57BL/6. C57BL/6 mice are normally $\underline{\text{Gus}}^b$ which produces high β -glucuronidase levels in adult tissues. $\underline{\text{Gus}}^h$ produces low β -glucuronidase levels. $\underline{\text{Gus}}^{h/b}$ heterozygotes had higher activity levels than $\underline{\text{Gus}}^h$ embryos beginning at the two-cell stage. This shows that the variation for embryonic activity levels and the paternal effect on activity levels indeed are due to differences at the $[\underline{\text{Gus}}]$ complex.

Multiple gene variation (or unspecified number) and the timing of paternal expression

Muggleton-Harris and Johnson (1976) used a C3H anti-C57BL/10 spleen antibody to probe the cell surface expression of non-H-2 alloantigens during preimplantation. In the cross, C3H $\text{♀} \times$ C57BL/10, F_1 embryos showed paternal antigen expression at the six-to-

eight-cell stage.

Whitten and Dagg (1961) found a small paternal effect on the intermitotic period between the first and second cleavage between BALB/c-GN embryos and F_1 embryos (BALB/c♀ X 129 ♂). The timing of the first cleavage was not different.

Summary

The lethal gene studies suggest that some vital embryonic functions require transcription. The timing of paternal gene expression in the single gene and multiple (or uncertain) gene variation studies shows that transcription of these genes could be starting at the late two-cell stage. These genetic studies support the notion that the onset of RNA synthesis in late two-cell embryos and early four-cell embryos is an indication of gene expression in the cleavage stage mouse embryo.

Parthenogenetic development

Both diploid and haploid parthenotes are capable of development to the blastocyst stage (Graham, 1970; Graham and Deussen, 1974; Kaufman and Sachs, 1976; Van Blerkom and Runner, 1976; Witkowski, 1973a). More advanced development has also been reported (Kaufman, Barton and Surani, 1977; Witkowski, 1973b). The fine structure of parthenote blastocysts is virtually identical to normal blastocysts (Van Blerkom and Runner, 1976). These results suggest that the sperm is not required for normal cleavage stage development. The variable response of eggs to different activation stimuli suggest that an incomplete fertilization response is the reason for the low percentage of parthenotes that reach the blastocyst stage rather than a

genetic incompetence.

Second, cleavage stage development is possible in the haploid state (Iles et al., 1975; Kaufman and Sachs, 1975; Kaufman and Sachs, 1976). But, loss of one chromosome from a haploid set does prevent cleavage to blastocyst (Kaufman and Sachs, 1975). OY embryos derived from the mating of XO females don't survive preimplantation (Morris, 1968). One complete haploid set of chromosomes is a necessity for preimplantation development.

Sperm mRNA

No detectable nuclear or cytoplasmic RNA remains associated with spermatozoa after the final steps of spermatogenesis. Although spermatocytes do synthesize RNA, the transcripts are shed along with the cytoplasm in the residual body (Daoust and Clermont, 1955). Monesi (1965) pulsed male mice with [³H]-uridine then prepared autoradiographs of testes two-to-three weeks after. Grains were found over spermatogonia and early-to-late spermatocytes, but late spermatozoa had no grains. Neither did late spermatozoa have grains over the nucleus with no chase during the transition of the spermatocyte to mature spermatozoan. Premkumar and Bhargava (1972) characterized RNA synthesis in bovine spermatozoa. All synthesis that did occur was associated with the mitochondria of the mid-piece. Protein synthesis also occurs in spermatozoa, but is also mitochondrial (Bragg and Handel, 1979).

Mouse sperm may have up to 3×10^{-14} mol per hr per sperm of β -galactosidase activity (data not shown). This is approximately ten-to-twenty percent the amount in eggs (see results).

One potential source of extrachromosomal sperm-derived developmental information is the sperm surface antigens. However, parthenotes seem to get through cleavage and cavitation. So, there is no specific requirement for these antigens known at this time.

MATERIALS AND METHODS

Animals

The mice used in this study were obtained from several sources. The inbred strains, C57BL/6J(B6), C57BL/10J (B10), CBA/J, SWR/J, and BALB/cJ were purchased from the Jackson Laboratory, Bar Harbor, Maine. The inbred strain, C3H/HeHa, and the random bred line, Ha/ICR, came from the West Seneca Laboratory, West Seneca, N.Y. C3H/St and the congenic strain, C57BL/6J.CBA-Trf^a Bgl-s^d (B6.CBA), were supplied by Dr. Verne Chapman of Roswell Park Memorial Institute, Buffalo, N.Y. Dr. Franklin Berger of Roswell Park Memorial Institute supplied the congenic strain, C57BL/10.C-H-7^b Bgl-e^a.

Dr. Chapman constructed the B6.CBA strain by transferring the Trf^a and Bgl-s^d alleles of CBA/J into the strain, C57BL/6J. The transfer involved six generations of backcrossing Trf^{a/b} Bgl-s^{h/d} heterozygotes to C57BL/6J, then four generations of brother-sister mating. Trf and Bgl-s map nine centiMorgans apart. C57BL/10.C-H-7^b Bgl-e^a (B10.C) was produced by direct transfer of the H-7^b allele from BALB/cJ to C57BL/10Sn with co-transfer of the Bgl-e^a allele of BALB/cJ occurring because it is only four centiMorgans from H-7 (Berger, Breen and Paigen, 1979).

Sources of Gametes

Mature ovulated eggs were obtained by inducing ovulation with pregnant mare serum gonadotropin (PMS) and human chorionic gonadotropin (HCG). Ovulated females were artificially inseminated to produce zygotes. Females were given an interperitoneal injection with 4-5 International units of PMS (Sigma, St. Louis, Mo., or Gestyl[®], Organon,

West Orange, N.J.) in 0.9% saline followed 48 hours later with an interperitoneal injection of 4-5 U.S.P. units of HCG (Ayerst Laboratories, Cleveland, Ohio) in 0.9% saline. Both PMS and HCG were maintained as one unit per μ l stocks in 0.9% saline. The HCG kept for a month at 4°C while the stock PMS kept indefinitely when frozen to -20°C. Twelve to thirteen hours after the last injection the females were artificially inseminated with 5×10^6 sperm on average (2×10^6 to 1×10^7 sperm was considered an acceptable range) in 0.05 ml of phosphate-buffered saline (PBS, pH 7.2). The preparation of sperm for insemination followed the procedure of West et al. (1977). To inseminate ten females, four males were killed and the cauda epididymis and vas deferens removed. These organs were minced with iridectomy scissors. The sperm were given 15 minutes to swim out of the tubules. The sperm suspension was mixed and drawn up into a 1 ml syringe equipped with a 1-1/2" 22 gauge needle which was blunted and bent to nearly a right angle 2 cm from its tip. Hormone-induced females were etherized just prior to insemination, suspended by the tail to present the vagina. The vagina was dilated with a blunt forceps and the 22 gauge needle inserted through the cervix. After injection of the sperm preparation, the vagina was plugged with a size number two dental cotton pellet (Richmond Dental Cotton Co., Charlotte, N.C.) soaked in 0.9% saline. The procedure is designed to deliver an optimal number of sperm into both horns of the uterus at the time ovulation is occurring. With this procedure, all females in a given experiment were inseminated within 20 minutes of each other. Fertilization within each group of females should have occurred synchronously. In comparing embryonic

development rates between strains or between embryos from females of the same strain, the mating behavior of the individual females and strain differences for mating behavior and sperm transport are reduced as additional variables.

Collection of unfertilized eggs

Females were induced to ovulate, then sacrificed 14 hours after the second injection, since by this time all eggs are in the oviduct. The dissected oviduct was put into PBS and the ampulla portion of the oviduct punctured with a dissecting needle to free the eggs. Adherent follicle cells were removed by incubating the eggs for 30 minutes in a PBS solution with 70 N.F. units of Bovine Testes hyaluronidase (Sigma) per ml. The eggs were rinsed and drawn rapidly through a narrow bore pipette to remove adhering follicle cells (see fig. 1, page 14).

Collection of embryos

Oviducts and uteri were dissected out of females and flushed with phosphate-buffered media (PBM, pH 7.2). The embryos were rinsed in the PBM.

Cell counts

For embryos with four or fewer cells, blastomeres can be easily counted in the intact embryo. Embryos with five to eight blastomeres were counted after freeze-thawing at one-hundred-fold magnification after assaying the embryos for β -galactosidase. More advanced stages required air-dried preparation of nuclei to obtain counts (Tarkowski, 1966). Slides were stained with Geimsa for 20 to 30 minutes, rinsed briefly with

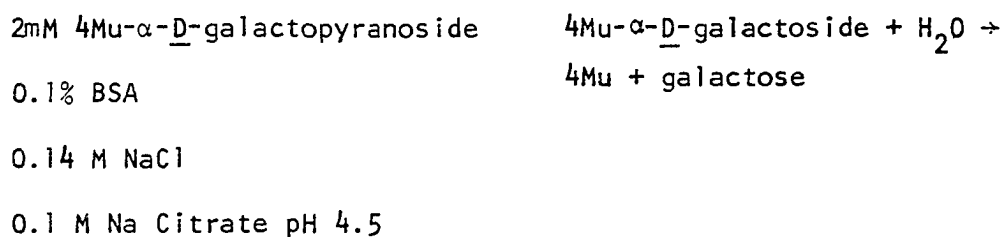
running tap water, then allowed to dry. For consistency with blastomere counts, metaphase spreads were scored as one cell, since one cell membrane would have surrounded the chromosomes in the intact embryo.

Enzyme assays

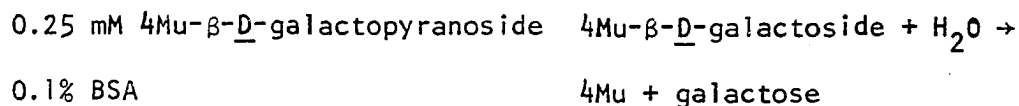
Single oocyte assays for α -galactosidase, β -galactosidase and β -glucuronidase were done according to the basic procedure of Wudl and Paigen (1974). The essence of this specialized assay is to increase the sensitivity of enzyme activity detection by performing it in nanoliter volumes. This reduces the background of substrate relative to the signal of product. The product signal is then collected by a microscope, sent through a photomultiplier and recorded on a chart. Free 4-methylumbelliferone in an alkaline buffer is highly fluorescent, with an excitation maximum of 365 nm and an emission maximum at 455 nm. The compound is available commercially as a conjugate of galactose (α and β forms) and glucuronic acid (Research Products International Corp., Elk Grove, Ill.). The compounds are non-fluorescent. The reaction mixtures for the activity assays are:

α -galactosidase -

reaction:



β -galactosidase -



0.14 M NaCl

0.1 M Na Citrate pH 4.7

β -glucuronidase -

3 mM 4Mu- β -D-glucuronide

4Mu- β -glucuronide + H₂O →

0.1% BSA

4Mu + glucuronic acid

0.14 M NaCl

0.1 Na Acetate pH 4.5

(Wudl and Paigen, 1974; Paigen et al., 1976; Adler, West and Chapman, 1977).

The assay procedure

Oocytes were rinsed in PBM, rinsed once in reaction mixture, then suspended in reaction mixture. The oocytes and reaction mix were drawn into the tip of a polyethylene tubing pipette and deposited on a microscope slide under a layer of mineral oil (USP 35, gift of the Amoco Oil Company) approximately 2 mm deep. The volumes of the individual drops ranged generally from 2 to 10 nl. The slide was placed in a gas-tight metal bottom box. The box was gassed with dry N₂ or CO₂, then placed alternately on dry ice and a warming plate three times to freeze-thaw the drops. After incubation at 37°C for 45-60 minutes, the slide was removed and placed in a desiccator along with two ml of triethylamine (TEA). The vapors of the TEA diffuse into the oil and into the drops raising the pH to 10. This stops the enzyme reaction and ionizes the 4-MU product, making it highly fluorescent. The reaction product was measured using a Phlum-illumination system to bombard the drops with incident light at 365 nm and collect the fluorescent emission. The emission beam was passed through narrow

band pass filter (Baird-Atomic) and sent to the photomultiplier. The sensitivity of the assay was 5×10^{-16} moles of 4-Mu. Blanks for the assay were empty drops. Standards were 4-Mu at 1×10^{-5} M in sodium citrate buffer (pH 4.5). Previously, Wudl and Paigen (1974) showed that freeze-thawing was necessary to start the reaction, that the fluorescence was proportional to both time and amount of enzyme and that the signal is independent of drop size for embryos.

Throughout the entire study, B6 oocytes were assayed for activity and provided an internal biological standard.

In the β -galactosidase assay, a special problem arose because the substrate has a low solubility. A late stage embryo could exhaust the supply of substrate in less than forty-five minutes. Forty-five minutes is the minimum time in which reliable assay results can be achieved. Using the dye phenol red, which turns from yellow to red around pH 6 to 7, I determined that it took from 5 to 15 minutes for the TEA to diffuse into the drops and raise the pH to 7 where the enzyme reaction is effectively stopped (see fig. 5, page 50). This much variation sometimes occurred in different drops on the same slide. To circumvent the problem of assaying late stage embryos, β -galactosidase assays for those embryos were run in μ l drops for two hours. The assay was started in the same way as the nl assay and ended by injecting 0.15 μ l of a solution of saturated sodium carbonate into the drops. Thirty drops can be injected in two minutes. Both assay procedures give comparable results. The μ l drop assay is linear for 2 hours and the fluorescence is proportional to the number of embryos in the drops (see fig. 2, page 42).

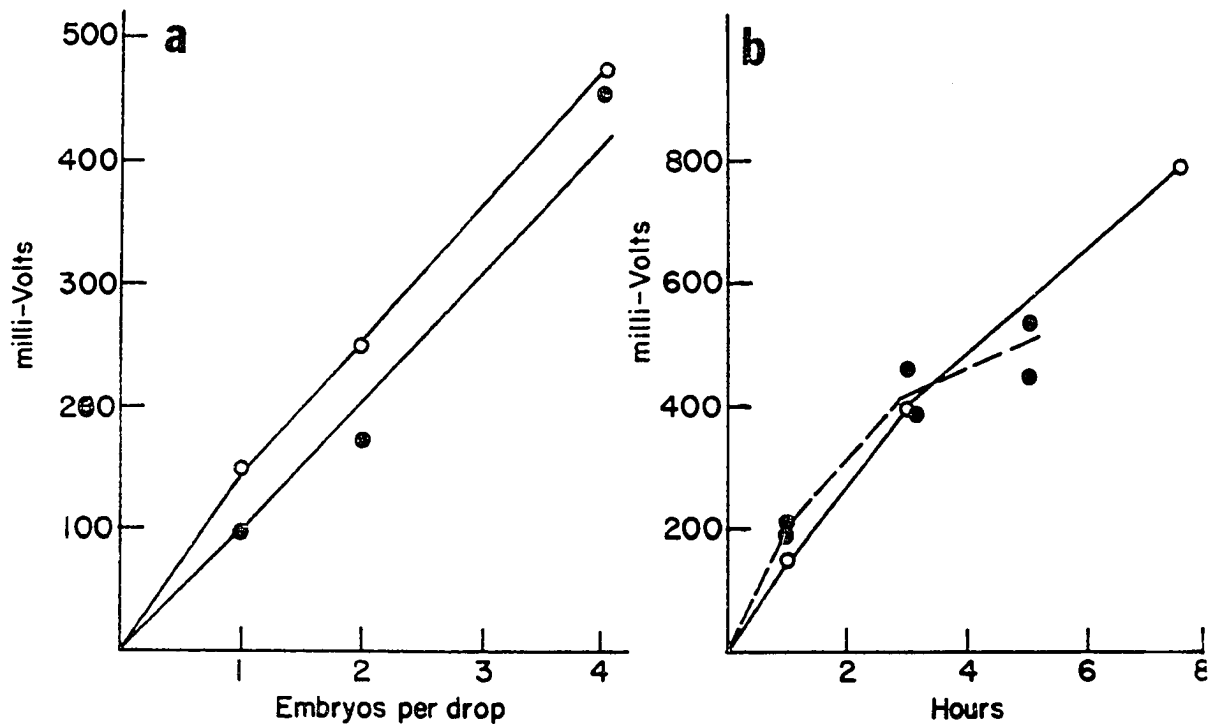


Figure 2. Assay of preimplantation mouse embryos for β -galactosidase using a μ l volume assay [(a) Eighty-four hour old SWR/J strain pre-implantation embryos were assayed for two hours with one, two or four embryos per μ l drop of substrate mixture. The individual points in the figure represent the mean milli-Voltage of three drops. The open circles are the result obtained from one group of females inseminated together, the solid circles, embryos from a second group of females. (b) SWR/J strain blastocyst were assayed one embryo per drop, for one, three, five, and eight hours. The open circles represent the mean milli-Voltage per embryo for three embryos. The solid circles are the milli-Voltage of single embryos from a different group of females].

The μ l assay was used for any strain and stage combination that showed a 10% total substrate consumption in the nl drop assay.

Immunosurgery

The separation of ICM from the embryo was accomplished by immunosurgery (Solter and Knowles, 1975). ICMs were compared to whole embryos obtained from the same females.

Embryos for ICM isolation were stripped of their zona pellucidas with a one-minute or less treatment in acid Tyrodes solution (pH 1.0). The remaining embryos were not treated with acid Tyrodes but placed immediately in PBM and 10% fetal calf serum. The naked embryos were rinsed twice in PBM with 10% fetal calf serum. They were then incubated in PBM plus 20% rabbit anti-mouse thymocyte IgG solution (Cappel Laboratories, Cochranville, PA) for thirty minutes. After two rinses in PBM, the embryos were suspended in 10% complement solution (1/10 dilution of Guinea pig sera, Gibco, Grand Island, N.Y.) for thirty minutes. By this time, the outside cells are lysing and can be clearly separated from the ICM by drawing and expelling them from a narrow bore pipette (see figs. 3 and 4, page 44).

The extent of outside cell contamination was determined by first incubating the intact embryos in the rabbit anti-mouse IgG, then after rinsing the embryos, incubating them in a solution of fluorescein conjugated goat anti-rabbit IgG (Cappel Laboratories) for thirty minutes, according to the scheme of Handyside (1978). After rinsing the embryos, the outside cells were lysed. These ICMs and unlysed embryos were then examined under fluorescence microscopy for the presence of

Figure 3. Indirect immune fluorescence of Ha/ICR embryos [Eighty-four hour old blastocysts were incubated for thirty minutes in Rabbit anti-mouse thymocyte IgG, rinsed, then incubated for 30 minutes in fluorescein-conjugated Goat anti-rabbit IgG. A is a photograph of two blastocysts. B is a fluorescence image of the same blastocyst].

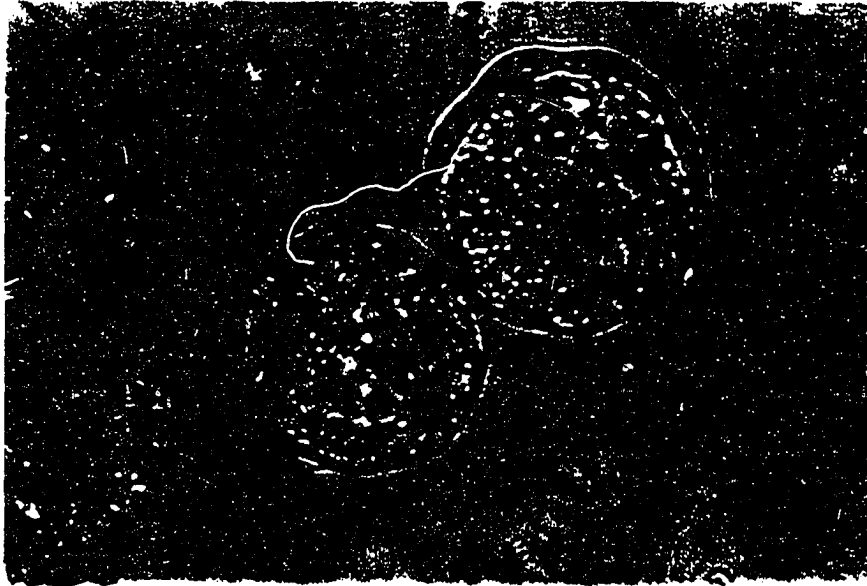
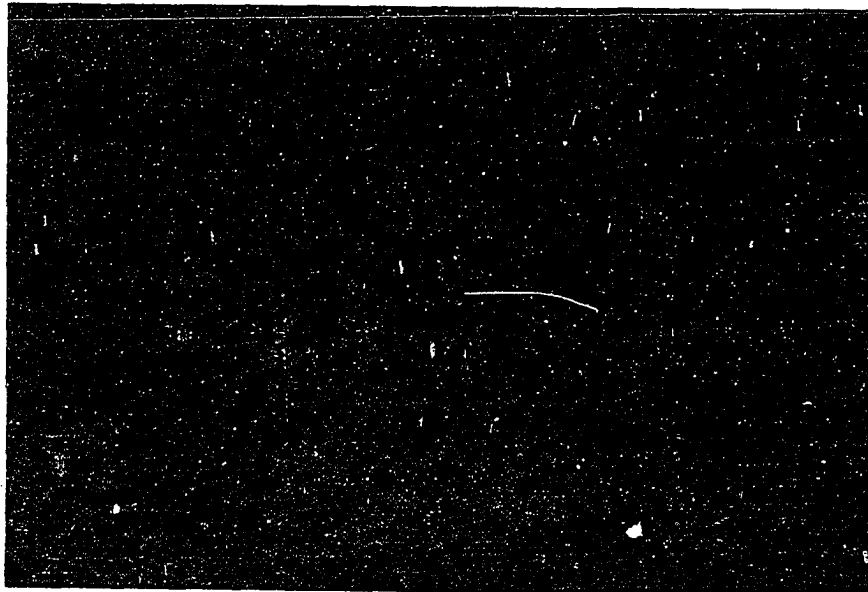


Figure 4. Indirect immune fluorescence of Ha/ICR embryos and inner cell masses obtained from intact blastocysts in immunosurgery [A is a photograph of two intact embryos (indicated by arrows) and five or six inner cell masses. B is a fluorescence image of the same group of embryos and inner cell masses. The intact embryos (arrows) are fluorescent showing the presence of intact outside cells still bound with fluorescein-conjugated Goat anti-rabbit IgG. One ICM is contaminated with outside cells (asterisk) but the rest are free of cells].



intact cells with the fluorescein label (see figs. 3 and 4, pages 44 and 46).

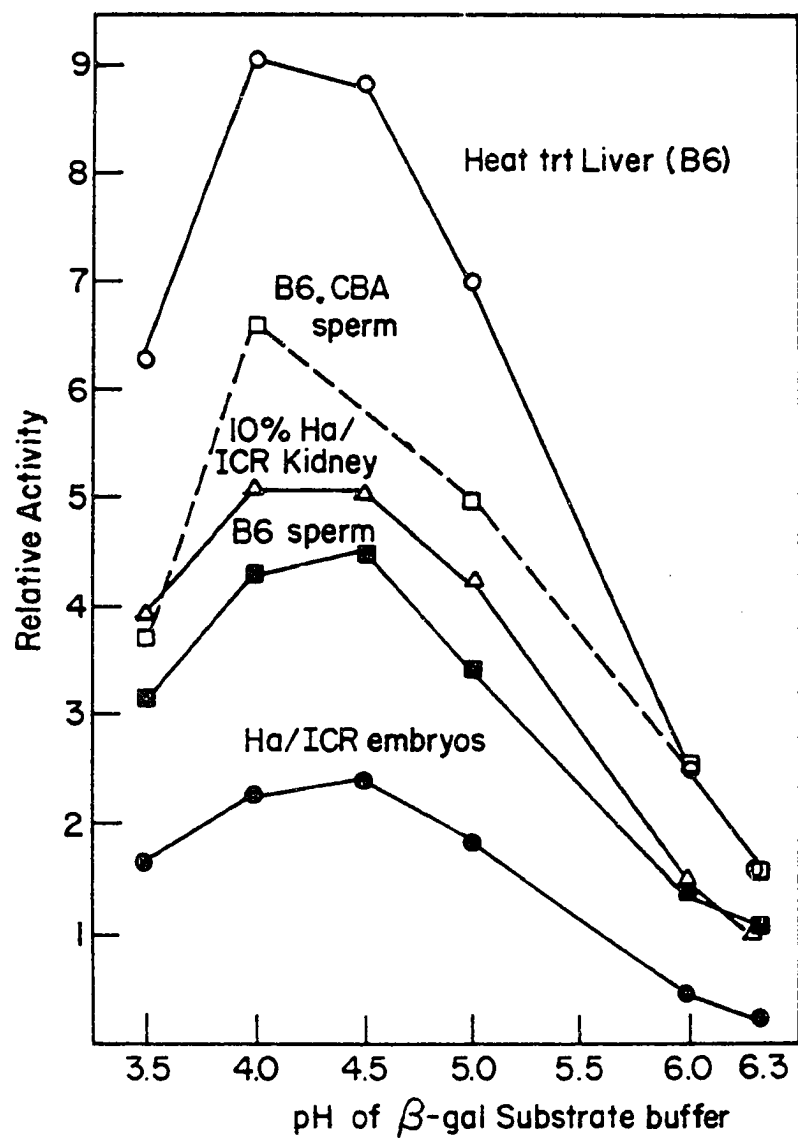
For assays and cell counts, ICMs were handled as intact embryos except that all glassware was silicone-treated and fetal calf serum was included in all solutions except the assay mixtures.

RESULTS

β-Galactosidase Activity in Sperm, Eggs
and Cleavage Stage Embryos

Epididymal sperm, ovulated eggs, and preimplantation mouse embryos all had a hydrolytic activity toward 4-methylumbelliferyl-β-D-galactopyranoside at pH 4.5. The pH optimum of the activity is 4.0-4.5 and shows the same sensitivity as liver and kidney activity (see figure 5, page 50). Since freezing and thawing of the cells is required to detect the activity, it is most likely intracellular. Figures 6-9 and table 11 (page 51 and page 85) show the activity levels of β-galactosidase in eggs and preimplantation embryos for various strains of inbred mice. Sperm activity levels are about $1.5-3.0 \times 10^{-15}$ mol/hr/sperm when measured in the strains B6 and B6.CBA, about ten to twenty percent of the egg values. Cleavage of the embryo to the two-cell stage is accompanied by a slight decrease in activity levels. Thereafter, the activity level per embryo increases logarithmically. At 84 hours post-insemination, activity levels are about twenty-five to fifty times the two-cell stage level. This represents no increase per cell to a two-fold increase per cell. The large increase in embryonic activity levels probably reflects new synthesis of enzyme. A comparison of the oocyte and embryonic activity levels between the various inbred strains surveyed shows a good deal of variation. Unfortunately, there is also a good deal of variation between strains for cell number per embryo after the four-cell stage (see table 5, page 72). This indicates that embryos of different strains develop at different rates.

Figure 5. pH optimum of β -galactosidase activity in a variety of mouse tissues when assayed with the 4-methylumbelliferyl β -galactopyranoside substrate [The buffer for the entire pH range was 0.1 M sodium citrate - citric acid. Heat trt liver-liver homogenate heated to 56⁰ C for one hour at pH 5 prior to assay. The individual curves were offset so the shape of all the curves would be clear. The relative activity area provides a guide to estimate the proportionate activity each tissue's enzyme has a particular pH].



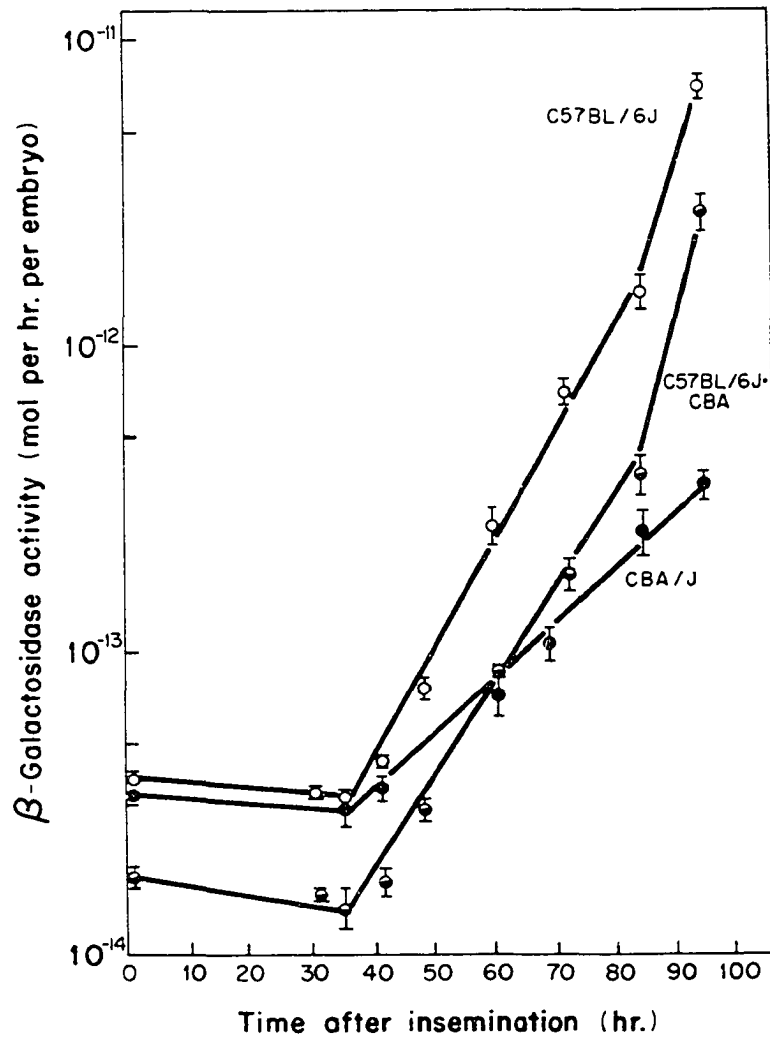


Figure 6. β -galactosidase enzyme activity levels of the pre-implantation embryos of C57BL/6J, CBA/J and a congenic inbred strain, C57BL/6J.CBA-Trf^a Bgl-s^d [The lower activity level of CBA/J compared to C57BL/6J.CBA may be due to the slower cleavage rate of CBA/J. The standard error of the mean is indicated by the vertical line. Each point is the average of six to twenty individual embryo assays].

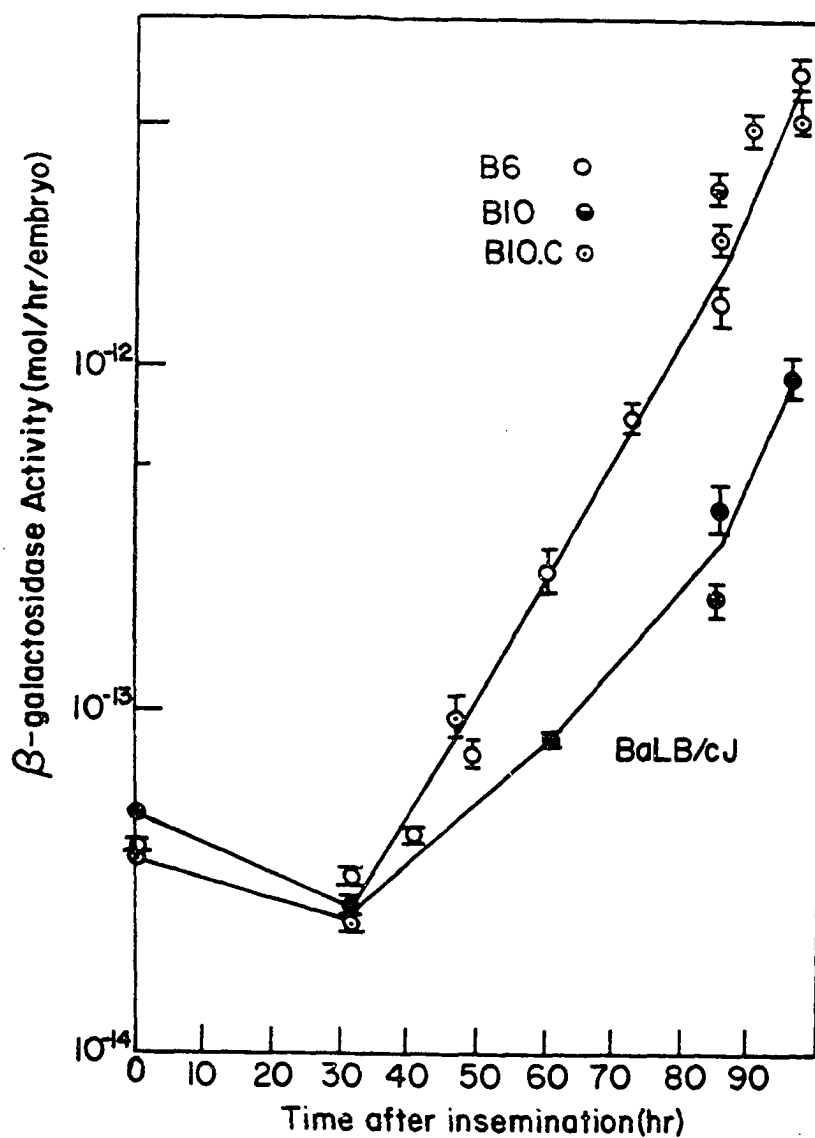


Figure 7. β -galactosidase enzyme activity levels of the pre-implantation embryos of C57BL/6J(B6), C57BL/10J(B10), BALB/cJ and a congenic inbred strain C57BL/10Sn.C-H-7^b Bgl-e^a [derived by the transfer of the H-7^b and Bgl-e^a alleles of BALB/cJ into B10].

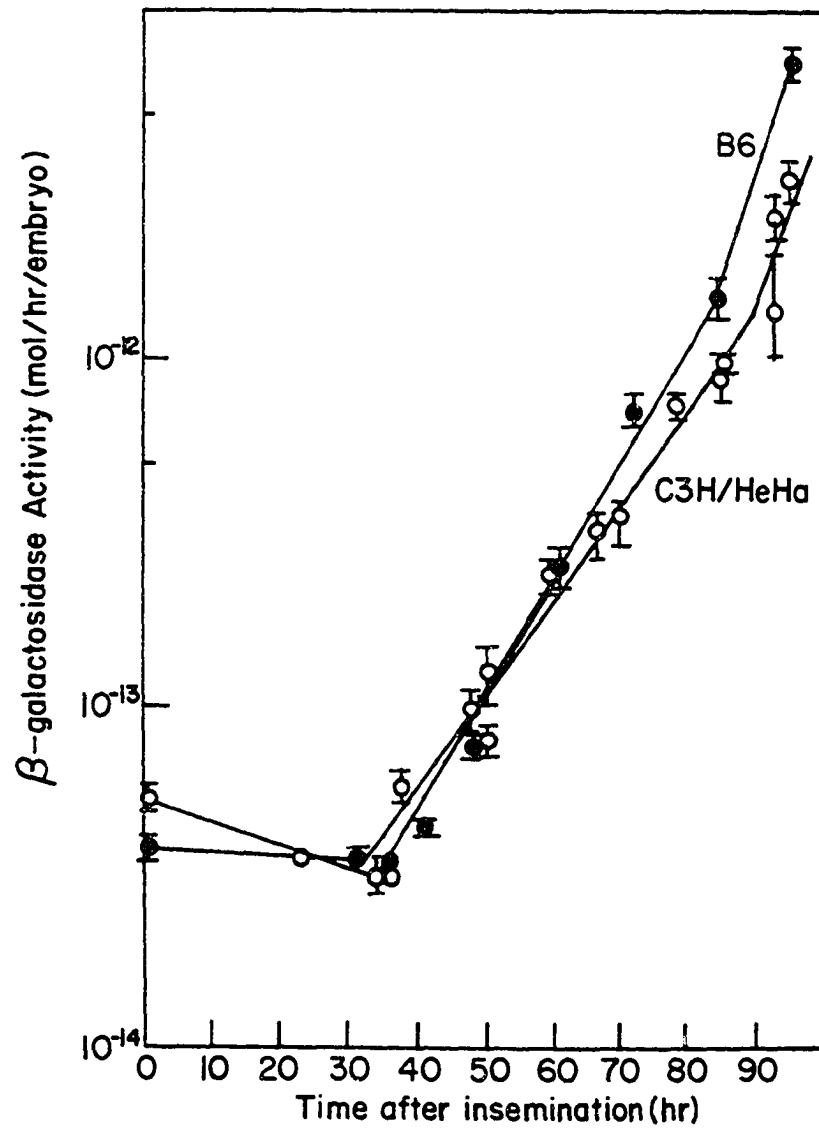


Figure 8. β -galactosidase enzyme activity levels of the pre-implantation embryos of C57BL/6J(B6) and C3H/HeHa.

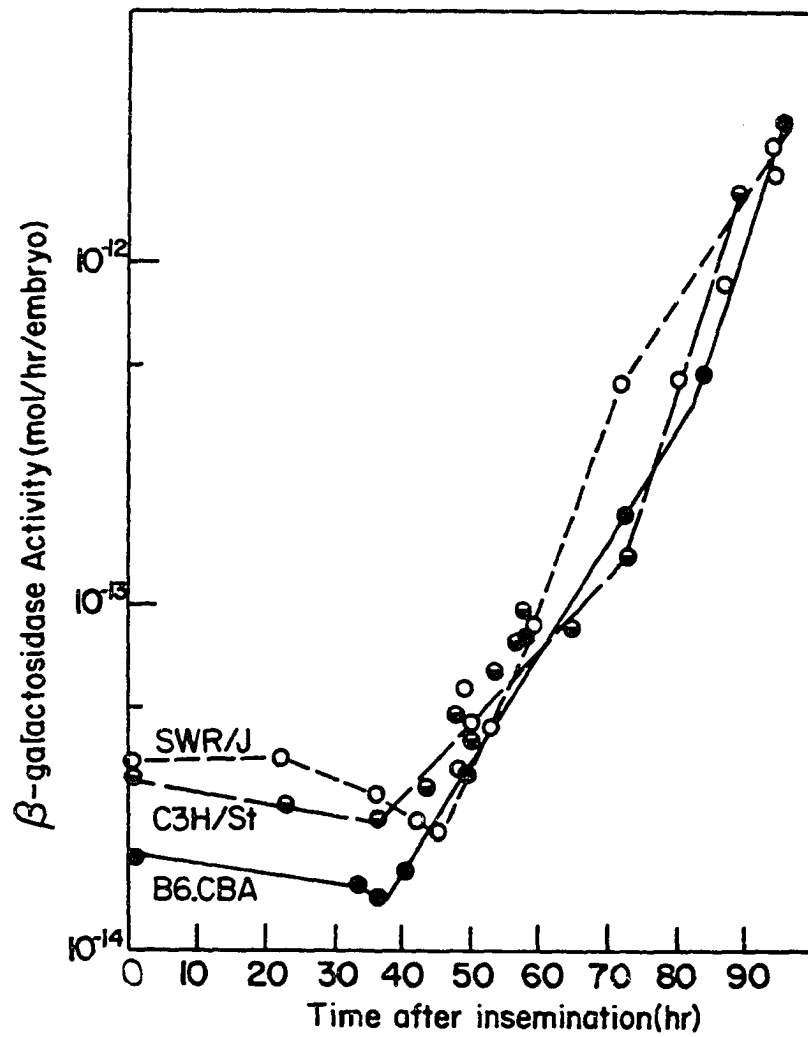


Figure 9. β -galactosidase enzyme activity levels of the pre-implantation embryos of SWR/J, C3H/St and C57BL/6J.CBA-Trf^a Bgl-s^d (B6.CBA).

Statistical Analysis of the Data

The oocyte samples and two-cell embryo samples approximate normal distributions with modal values near the arithmetic mean (see figs. 10-14, page 56). Differences in the means values of such samples were evaluated by the Student's t-test. This situation persists up to 40 hours post-insemination. After this time, individual samples often show no centrally-placed modal value. The coefficient of variation (SD/Mean) doubles or triples. These changes in sample parameters accompany the beginning of activity level increases at about 40 hours post-insemination. The increase in the coefficient of variation is due to the exponential nature of the activity level increase. Examining the relationship between activity levels and cell number for the second cleavage shows some cases where embryos that have cleaved have a higher activity than those that haven't. This is not a clear distinction. As shown in figure 12, SWR/J four-cell embryos between 45 and 49 hours have more activity than two-cell embryos in the same sample. This is also true for the C3H/HeHa 36-hour sample (see fig. 14). However, none of the other samples from other inbred strains show such a tendency (see figs. 10, 11 and 13). The SWR/J and C3H/HeHa examples indicate that individual embryo cleavage rate variation exist even at early stages. This variation might contribute to activity level variation in samples.

In most cases, the coefficient of variation is a constant after the initial increase that occurs in four-cell embryos. In order to test these differences between the means of samples whose standard deviations are proportional to the mean, the data were transformed into

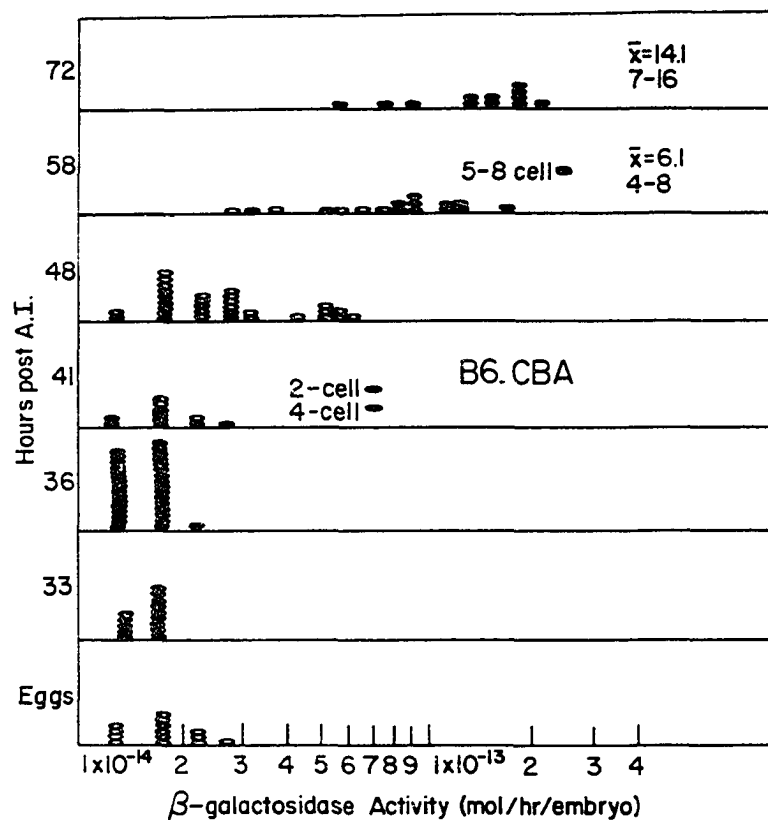


Figure 10. Individual embryo and oocyte β -galactosidase activity assays for the inbred strain C57BL/6J.CBA-Trf^a Bgl-s^d [Each panel represents the results of assaying embryos from three or four females inseminated at the same time. \bar{X} is the mean cell number of the sample embryos. The range of cell numbers is also given].

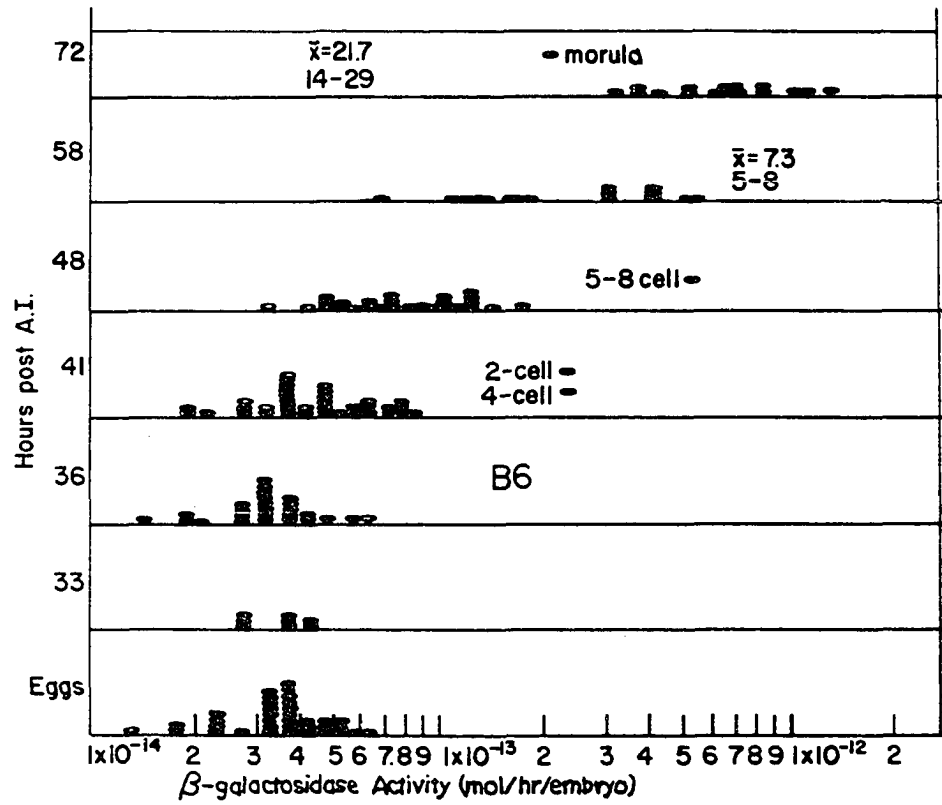


Figure 11. Individual embryo β -galactosidase activity assay for the strain C57BL/6J.

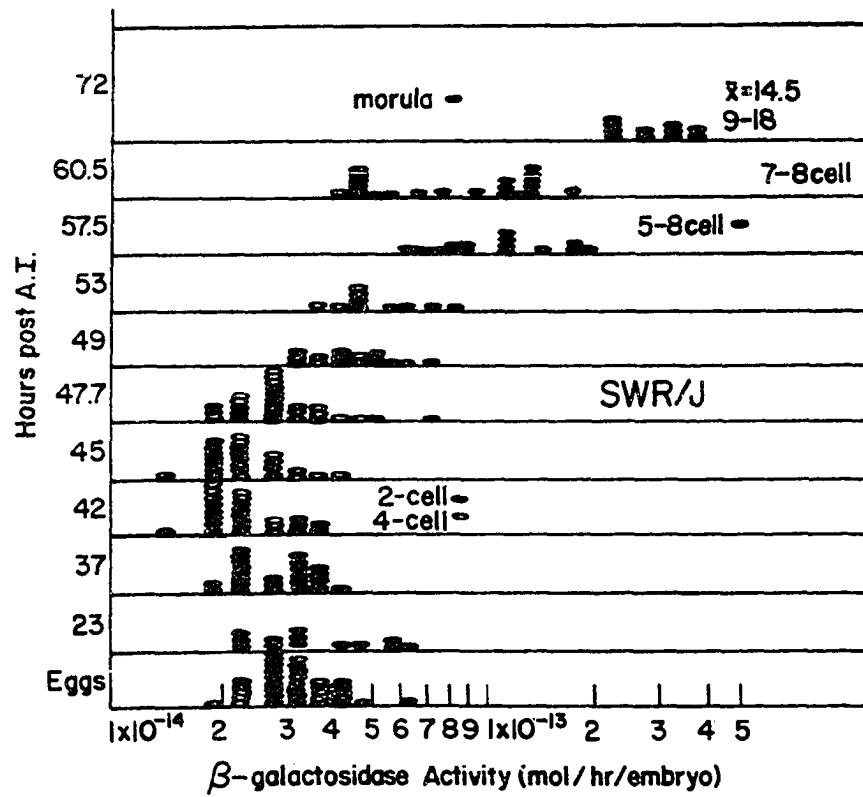


Figure 12. Individual embryo β -galactosidase assay for the strain SWR/J.

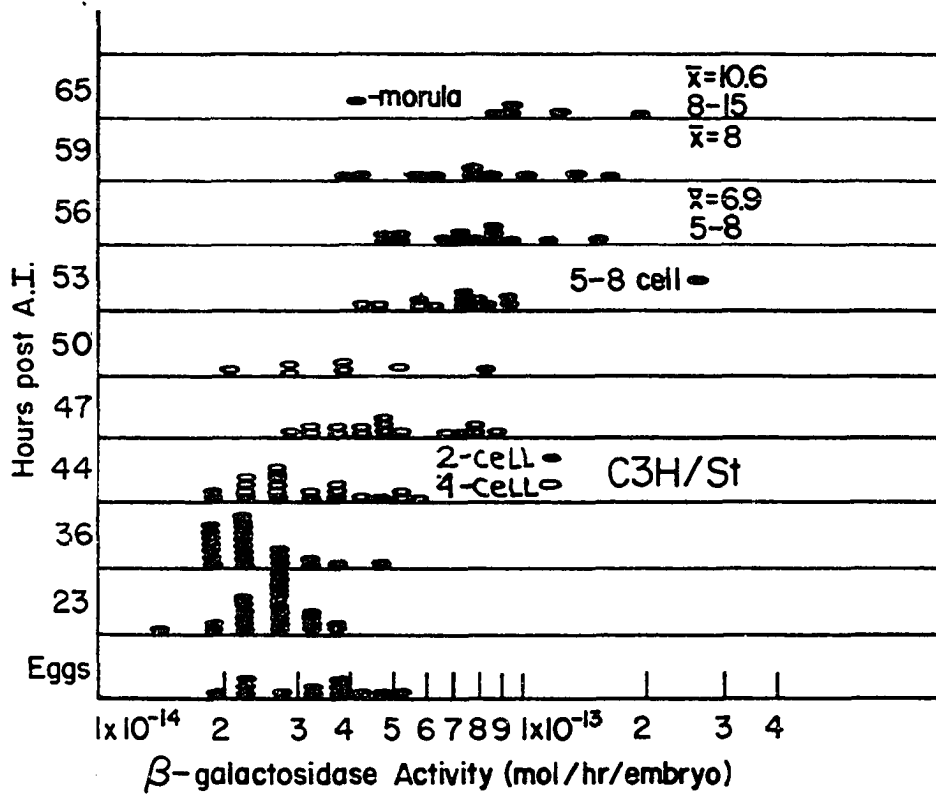


Figure 13. Individual embryo β -galactosidase activity assays for the strain C3H/St.

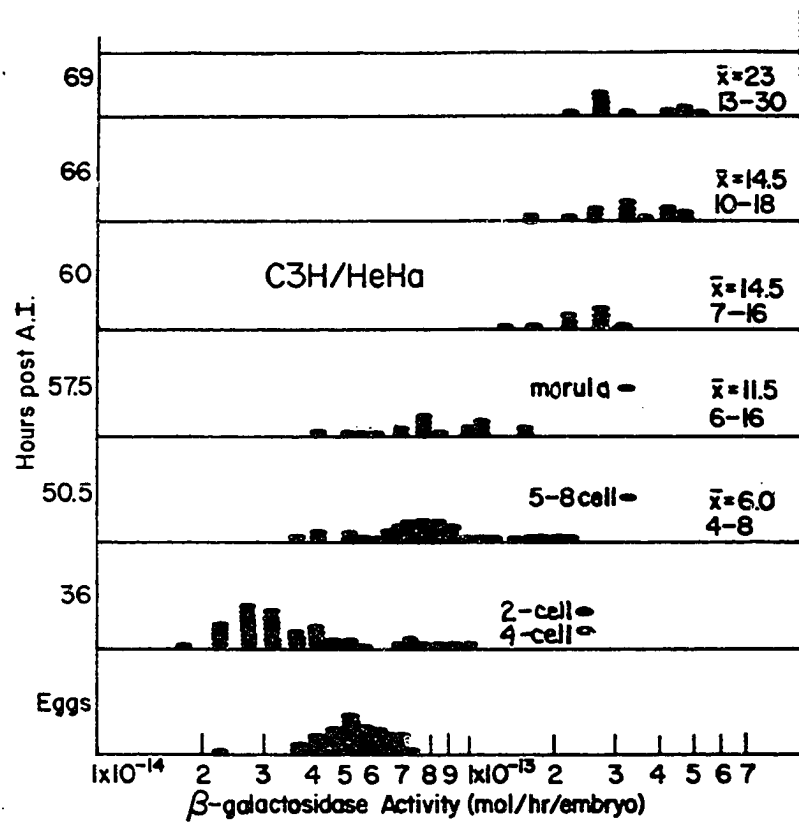


Figure 14. Individual embryo β -galactosidase activity assay for the strain C3H/HeHa.

base 10 logarithms (Snedecor and Cochran, 1976). This accomplishes two things. First, the transformed activity data can be fitted by a straight line between 36 and 84 hours post-insemination (see figs. 6-9). Secondly, the Student's t-test should now be applicable to test differences between sample means. The evaluation of sample differences using the non-parametric Whitney-Mann Rank test on untransformed data and an evaluation of sample mean differences using the Student's t-test on \log_{10} transformed data for B6.CBA embryo activity levels versus the F_1 (B6.CBA ϕ X B6 σ^1) give identical results. Since the t-test is much easier to apply, it was used for the remainder of the statistical analysis. Cell number data were analyzed, after conversion to \log_{10} , by the Student's t-test.

[Bgl] Haplotypes and Embryonic Activity Levels

Do [Bgl] complex haplotypes determine the preimplantation β -galactosidase activity level variation between inbred strains of mice? I would expect only the Bgl-s component of the [Bgl] complex to cause between strain variation for β -galactosidase activity levels in embryos. Activity level variation caused by Bgl-s would be on the order of a 1.5 to 2.5-fold difference. There are only two alleles of Bgl-s, so if between strain variation is due to Bgl-s alone, then the activity levels of mouse embryos should fall into two classes in concordance with the adult brain activity levels of each strain.

Does the maternal or embryonic [Bgl] complex haplotype determine embryonic activity levels? Again this question is approached with the expectation that the Bgl-s component of [Bgl] would cause the variation in activity levels between a group of homozygous embryos and a group of het-

erzygous embryos. Bgl-s expression is additive in heterozygous mice. The action of Bgl-s is associated with enzyme synthesis. Since embryo activity levels begin increasing at the time of the second cleavage, this predicts that heterozygous mouse embryo activity levels will have maternal-type embryo activity values at the second cleavage then activity levels intermediate to maternal-type and paternal-type embryos as soon as the increase in total activity levels dilutes out ova enzyme. The difference in activity levels between homozygous and heterozygous embryos should be 1.25 to 1.75-fold by this time.

These questions were tested as follows:

First, the preimplantation embryonic activity levels and rates of enzyme activity increase between B6 and the congenic inbred strain, B6.CBA, were compared. In theory, these two strains differ genetically only for a ten centiMorgan length of chromosome nine which includes the Trf locus and the [Bgl] complex. These two inbred strains were crossed and the F₁ embryo activities examined to see if the genotype of the embryo determined the activity level of β -galactosidase.

The second test was a comparison between several inbred strains to see if [Bgl] haplotypes alone explained the β -galactosidase activity level variation between the embryos of these strains.

The cytodifferentiation of trophectoderm and ICM occurs during the preimplantation period. The expression of trophectoderm or ICM specific genes may obscure total embryonic activity level variation due to [Bgl] complex variation. A third test involved

separating the ICM cells from trophectodermal cells of the embryo to compare their cellular activity levels.

β -Galactosidase variation between B6 and B6.CBA preimplantation embryos

A comparison between B6 and B6.CBA strain preimplantation β -galactosidase levels appears in figure 6. B6 embryos enter the second cleavage (36 to 41 hrs. in fig. 6) with twice the activity levels of B6.CBA embryos. This difference is due to a carryover of enzyme from the ova (see figs. 10, 11). By 84 hours after insemination, B6 embryos have almost four times the activity level of B6.CBA embryos. The difference at all stages is significant (Student's t-test $p < 0.01$).

Quite unexpectedly, the comparison of B6.CBA and B6 cleavage rates shows them to differ significantly (see table 3, page 46). There are two reasons for examining the cleavage rates and β -galactosidase activity level differences. First, if the differences in cleavage rates were large enough relative to the differences in activity levels, then differences in overall embryonic developmental rate might be the cause of any activity level difference between strains at a given time after fertilization. Also, a large difference in cleavage rate between B6 and the congenic B6.CBA could indicate that a significant amount of the CBA/J genome (see CBA/J and B6 in table 5, page 50), in addition to the segment on chromosome nine including Trf and [Bg1], was still present in the congenic. If this were true, then loci other than the [Bg1] complex might be contributing to the preimplantation β -galactosidase activity differences between these strains. Second, when the preimplantation embryos of other inbred strains are examined, it will be necessary to have an estimate of just how much activity level

Table 3 Comparison of mean cell number per embryo during the preimplantation stage for C57BL/6J, B6.CBA-Trf^aBgl-s^d and F₁ (B6.CBA ♀ X B6 ♂)

Time after insemination (hr.)	Mean cell number per embryo ^a		
	<u>C57BL/6J</u>	<u>B6.CBA</u>	<u>F₁</u>
36	2.0	2.0	2.0
41	2.5 [±] 0.1 ^b (54) ^c	2.2 [±] 0.2 (17)	2.1 [±] 0.04 (57)
48	4.7 [±] 0.2 (29)	3.8 [±] 0.1 (41)	4.0 [±] 0.0 (41)
58	7.3 [±] 0.4 (13)	6.1 [±] 0.4 (19)	6.9 [±] 0.3 (39)
72	21.7 [±] 2.4 (10)	14.1 [±] 1.7 (9)	14.5 [±] 2.5 (8)
96	59.6 [±] 6.0 (9)	56.0 [±] 5.0 (7)	-

^aAt 36, 41 and 48 hr., blastomeres of intact embryos were counted. For later stages the air-dry method of Tarkowski (1966) was used.

^bStandard error of the mean.

^cNumber of embryos counted.

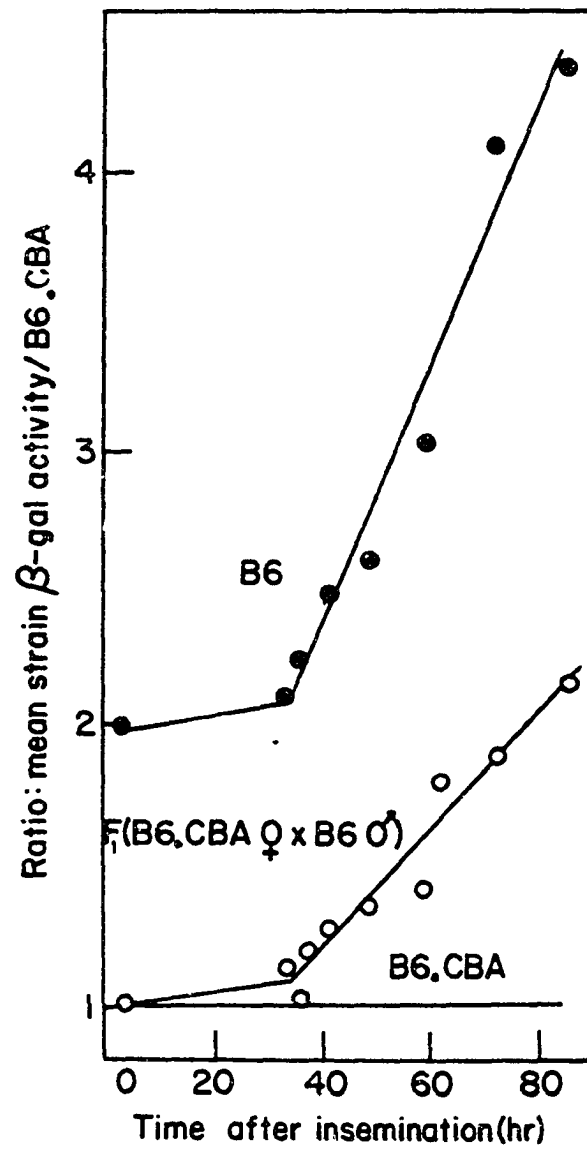
variation occurs between B6 and B6.CBA embryos due to [Bgl] haplotype variation alone. Table 7 (page 76) compares the cellular activities of B6.CBA and B6 embryos at several time points after insemination. This comparison shows B6 and B6.CBA levels differing from two-fold at the two-cell stage and a 3.0 to 3.2-fold difference at later times. This is the same result as that obtained by comparing the best fit lines from a linear regression analysis of the \log_{10} activity levels on mean cell number per embryo for B6 and B6.CBA.

Embryonic genotype and embryonic β -galactosidase levels

The cross, B6.CBA ♀ X B6 ♂ , produced a population of preimplantation embryos that cleaved at the same rate as B6.CBA embryos (see table 3). The activity levels of the two-cell embryos of the F_1 and B6.CBA are almost the same, but by 84 hours post-insemination, the F_1 has about two-fold higher levels (see fig. 15, page 66). The reciprocal F_1 has two-cell activity levels like B6 embryos and half the 84-hour activity level of B6 embryos (see fig. 16, page 68). I don't have cell number data on this F_1 . The 84-hour differences between B6 and the F_1 embryos and B6.CBA are significant (Student's t-test, $p \leq 0.05$). Since the F_1 (B6.CBA ♀ X B6 ♂) does cleave like B6.CBA and still has a higher activity level at 84 hours, the variation between B6.CBA and B6 embryos is attributable to [Bgl] complex variation.

The F_1 data answer three additional questions about the expression of the [Bgl] complex. First, the lack of maternal haplotype dominance in the embryonic activity levels shows that the activity is intrinsic to the embryo and not absorbed from the environment. Second, the effect of the paternally-derived [Bgl] haplotype on activity levels

Figure 15. The relative mean β -galactosidase activity levels of B6, B6.CBA and F_1 (B6.CBA \varnothing X B6 σ^7) preimplantation stage embryos [The figure combines data from two experiments. In one experiment, embryos of all three types were collected and assayed simultaneously. In the second experiment F_1 and B6.CBA embryos were collected and assayed together. The ratios are derived from mean activity values of each set of simultaneously-assayed embryos].



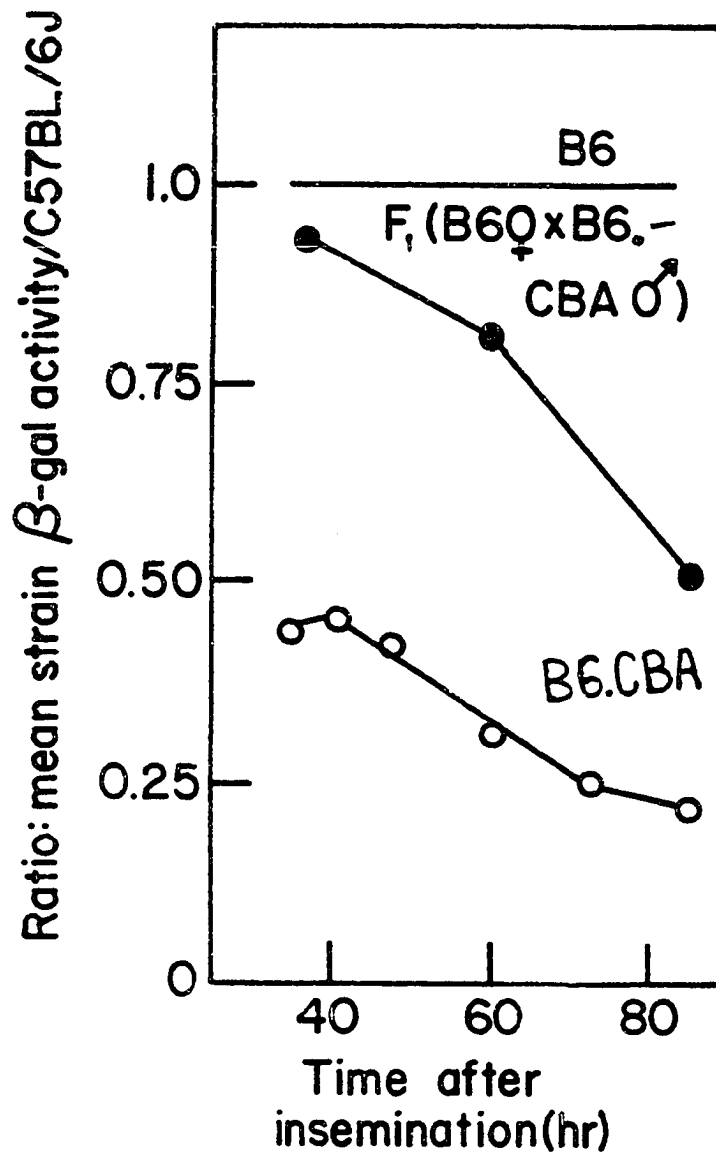


Figure 16. The relative mean β -galactosidase activity levels of B6, B6.CBA, and F_1 (B6 ♀ X B6.CBA ♂) preimplantation embryos [B6 and F_1 embryos were assayed simultaneously and the mean activity values from each assay were used to derive the ratio. The relative activities shown for B6.CBA are from the previous figure].

occurs over a period when activity levels are increasing. This suggests that the higher activities are, in part, a consequence of Bgl-e transcription beginning at the early four-cell stage (see figs. 6 14 and 15. The activity level differences between B6.CBA and the F_1 (B6.CBA ♀ X B6 ♂) are significant from 48 hours to 84 hours post-insemination (Student's t-test). Third, the F_1 data were again examined to see if they could help distinguish the amount of variation caused by [Bgl]. In the F_1 (B6.CBA ♀ X B6 ♂), the cleavage rate is the same as B6.CBA. If the cleavage rate difference between B6.CBA and B6 embryos is indicative of additional genetic differences that might affect β -galactosidase expression, their effects might be reduced in the F_1 . The [Bgl] complex differences under consideration are rates of enzyme synthesis differences. The proportion of enzyme activity level increases per embryo exceeds the changes in gross protein synthetic rates over the preimplantation period (Brinster, Wiebold and Brunner, 1976; Epstein, 1975). This suggests that β -galactosidase has a longer half-life than most proteins in the embryo, which Brinster, Wiebold and Brunner (1976) estimate is 12 to 18 hours. If the enzyme half-life is on the order of a day or longer, then rates of accumulation of enzyme for intervals of 12 to 24 hours may be a good estimate of rates of enzyme synthesis. Enzyme activity changes for successive periods between 36 and 84 hours of development are shown in table 4 (page 70) for B6.CBA and F_1 embryos. A comparison of these increases in β -galactosidase activity shows that the F_1 has a 1.5 to 2-fold greater increase during this time. A similar comparison was made between B6 and reciprocal (B6 ♀ X B6.CBA ♂) F_1 embryos. A similar 1.5 to 2-fold difference

Table 4 Comparison of β -galactosidase rates of activity level changes between B6.CBA and F_1 (B6.CBA ♀ X B6 ♂) - B6 and F_1 (B6 ♀ X B6.CBA ♂) preimplantation stage mouse embryos

Interval (hr. after insemination)	Total activity level increase(mol/hr/embryo)		Ratio (F ₁ /B6.CBA)
	B6.CBA	F ₁ (B6.CBA ♀ X B6 ♂)	
Exp. 1			
36-41	0.25 X 10 ⁻¹⁴	0.7 X 10 ⁻¹⁴	2.8
41-48	1.5 X 10 ⁻¹⁴	2.6 X 10 ⁻¹⁴	1.7
48-58	5.0 X 10 ⁻¹⁴	7.0 X 10 ⁻¹⁴	1.4
58-72	1.0 X 10 ⁻¹³	2.0 X 10 ⁻¹³	2.0
Exp. 2			
37-60	3.0 X 10 ⁻¹⁴	6.0 X 10 ⁻¹⁴	2.0
60-86	3.3 X 10 ⁻¹³	6.6 X 10 ⁻¹³	2.0
	B6	F ₁ (B6 ♀ X B6.CBA ♂)	Ratio (B6/F ₁)
36-60	1.1 X 10 ⁻¹³	0.8 X 10 ⁻¹³	1.4
60-84	2.1 X 10 ⁻¹²	1.0 X 10 ⁻¹²	2.1

in activity levels occurs between F_1 and B6 embryos. Taken together, the combined estimate of F_1 embryo and homozygous embryo differences suggest a total three to four-fold difference for rates of β -galactosidase enzyme synthesis between the embryos of B6 and the congenic, B6.CBA.

β -Galactosidase activity variation between several inbred strains of mice

Several inbred strains of mice were examined to see if the [Bg1] haplotype was the only source of genetic variation that resulted in interstrain differences in β -galactosidase levels during preimplantation.

Evaluating the embryonic β -galactosidase activity phenotypes of the embryos in these strains presented a more difficult problem than the comparison between B6 and B6.CBA. The cell numbers per embryo differ considerably between strains for any given time after insemination (see table 5). Linear regression equations of the cleavage rates beginning at the two-cell stage on show many significant differences among strains (see table 6, page 74). The intercept differences are ignored because they have no real meaning and because embryos of most strains cleave from two-cell to four-cell at about the same time (see table 5 and figs. 10-14).

If activity levels per cell were constant for all strains during cleavage, then interstrain comparisons would be simplified. But examination of table 7 shows that this is not the case. There are two direct ways to evaluate the activity level data in light of the problem. The first assumes that activity level increases occur according

Table 5 Comparison of cell numbers per embryo between inbred mouse strains

Time after insemination (hr.)	Mean cell number per embryo \pm SEM		
	<u>B6</u>	<u>B10.C</u>	<u>C3H/HeHa</u>
24	2.0	-	2.0
36	2.0	2.0	2.2 \pm 0.01
40-42	2.5 \pm 0.08	-	3.4 \pm 0.08
47-50	4.2 \pm 0.11	6.4 \pm 0.51	5.7 \pm 0.27
57-60	7.8 \pm 0.26	-	14.3 \pm 0.86
70-74	19.2 \pm 0.86	-	-
78-80	38.3 \pm 2.8	-	26.6 \pm 1.2
84-85	40.7 \pm 2.8	51.7 \pm 4.0	54.2 \pm 2.3
88-92	-	76.4 \pm 5.8	60.8 \pm 3.0
96	57.9 \pm 4.9	-	64.9 \pm 3.3

Table 5 (continued)

Time after insemination (hr.)	Mean cell number per embryo \pm SEM			
	<u>C3H/St</u>	<u>SWR/J</u>	<u>CBA/J</u>	<u>BALB/J</u>
24	2.0	2.0	-	2.0
36	2.0	2.1 \pm 0.1	2.0	-
40-42	2.7 \pm 0.21	2.3 \pm 0.10	2.8 \pm 0.30	-
47-50	4.0 \pm 0.1	3.5 \pm 0.06	3.8 \pm 0.10	-
57-60	7.2 \pm 0.24	6.4 \pm 0.31	5.7 \pm 0.22	-
70-74	19.8 \pm 1.44	15.0 \pm 0.63	10.9 \pm 1.1	-
78-80	38.7 \pm 6.2	17.8 \pm 2.5	-	-
84-85	47.8 \pm 2.8	53.1 \pm 4.9	28.6 \pm 2.3	29.2 \pm 4.5
88-92	47.4 \pm 4.0	-	-	-
96	-	-	35.0 \pm 7.2	-

Table 6 Comparison of cleavage rates between inbred mouse strains

Strains	Least squares regression line slope (β) ^a
C3H/St (1)	0.0285 (3,5,6 [*]) ^b
SWR/J (2)	0.0283
B6 (3)	0.0271 (5,6 [*])
B10.C (4)	0.0266
C3H/HeHa (5)	0.0264 (6)
B6.CBA (6)	0.0246 (7 [*])
CBA/J (7)	0.0204

^aThe regression equation was $Y = \beta X - \alpha$, where Y is hours after insemination and X is \log_{10} cell number per embryo.

^bcomparisons between strains for B. Asterisk indicates a significant difference ($P < 0.05$). Slopes (β) were compared pairwise being an analysis of variance with the F-test and to evaluate deviations from the regressions for pooled data and the separate regressions (Snedecor and Cochran, 1976).

to a clock schedule. This ignores cleavage rates and just compares strain differences at set times after insemination on a per cell basis. A second way assumes that the developmental program of the embryo follows cell divisions. In this case, total embryonic activity is compared between strains for embryos that have gone through a similar number of cell doublings. Also, both hours of development and cleavage can be combined by limiting strain comparisons to strains with embryos that cleave at the same rate. Table 7 compares strain levels on the first basis. Table 8 provides the second and third types of comparison. In table 8, the third comparison is made within columns for strains with an asterisk.

All three types of comparison confine the total between strain variation to the limits seen in the B6 and B6.CBA embryo variation, with the exception of the 96 hour post-insemination results.

A comparison of $[Bgl]^{D2}$, $[Bgl]^{B6}$ and $[Bgl]^C$ strains suggest three activity classes. At 84 hours, these would be 1.1 to 1.9×10^{-14} mol/hr/cell, 2.4 to 2.6×10^{-14} mol/hr/cell and 3.6 to 4.6×10^{-14} mol/hr/cell. This variation is similar to differences in activity levels among these strains in adult liver.

β -Galactosidase expression during the first differentiation event

If β -galactosidase is not expressed equally by cells of the ICM and trophectoderm, then differences in cleavage rates may be compounded by differences in the timing of differentiation and its effect on β -galactosidase levels. Examining ICM and trophectoderm strain variation separately also might aid in distinguishing the nature of genetic differences between strains. I separated ICM cells from whole embryos

Table 7 β -galactosidase activity per cell in the preimplantation stage embryos of nine inbred strains at various times after insemination

Strain	β -galactosidase activity/cell (mol/hr/embryo $\times 10^{14}$)					
	<u>36^a</u>	<u>48</u>	<u>60</u>	<u>72</u>	<u>84</u>	<u>96</u>
B6	1.7	2.0	2.5	3.3	3.6	12.5
B10	-	-	-	-	4.0	-
B10.C	1.4	2.3	-	-	4.6	4.8
BALB/cJ	1.3	-	1.4	-	2.6	2.4
C3H/HeHa	1.6	1.7	1.7	2.0	2.4	4.0
B6.CBA	0.7	0.8	0.9	1.1	1.1	4.5
C3H/St	1.2	1.1	1.1	1.0	1.0	2.2
SWR/J	1.2	1.1	1.1	2.1	1.9	1.7
CBA/J	1.5	1.2	1.2	1.1	1.1	1.1

^aHours after insemination.

Table 8 Comparison of inbred strain embryo β -galactosidase activity per cell between embryos of equal cell number for 2-cell to 56-cell embryos.

Strain	Activity per cell				
	<u>2 cells</u>	<u>3-4 cells</u>	<u>5-8 cells</u>	<u>9-14 cells</u>	<u>15-20 cells</u>
B6	1.7 ^a	2.0	2.5	-	3.1
B.10	-	-	-	-	-
B10.C	1.4	-	1.4	-	-
C3H/HeHa	1.6	-	1.8	1.8	-
BALB/cJ	1.4	1.6	-	-	-
B6.CBA	0.8	0.9	1.0	1.1	-
C3H/St	1.2	1.2	1.1	-	1.0
SWR/J	1.2	1.1	1.4	-	2.0
CBA/J	1.6	1.3	1.2	1.1	-

^a $\times 10^{14}$ mol/hr.

Table 8 (continued)

Strain	Activity per cell			
	<u>25-35 cells</u>	<u>36-41 cells</u>	<u>45-50 cells</u>	<u>51-56 cells</u>
B6	-	3.5 [*]	-	12.5
B.10	-	-	-	4.0 [*]
B10.C	-	-	-	4.6 [*]
C3H/HeHa	2.4	-	-	2.5 [*]
Balb/cJ	2.6 ^{*b}	-	-	-
B6.CBA	-	1.1 [*]	-	4.5
C3H/St	-	-	1.5 [*]	-
SWR/J	-	-	-	1.6 [*]
CBA/J	1.1 [*]	1.0	-	-

^bAsterisk indicates when the embryos in that strain were 84 hours old.

and assayed them to see if the first differentiation event had any effect on β -galactosidase levels. Ideally, some time should elapse between the differentiation event and measurement of the activity levels to allow for new synthesis and turnover to occur under the differential conditions. The exact timing of differentiation has not been determined in the mouse embryo. Johnson, Handyside and Braude (1977) have suggested that the acquisition of different properties by the ICM and trophectoderm over a span of time could mean that facets of differentiation are not triggered at a single point, but acquired gradually over time. With this caveat in mind, I decided to assay inside cells 10-12 hours after segregation of inside cells into the ICM begins. Table 9 (page 80) shows that for most strains, cavitation begins around 74 to 78 hours after insemination and is completed by 90 to 94 hours. Hatching from the zona pellucida and transformation of mural trophectoderm cells into giant cells with excess DNA begins for some strains at about 94 hours. I did not want to include embryos with giant cells in the analysis because it would be impossible to relate activity to cell numbers. So, I was constrained to sampling between 85 hours and 92 hours. Between 74 hours and 84 hours the inbred strains used doubled or tripled their embryonic activity levels. One cell doubling occurs during this time. This amounts to either no net increase per cell or a 1.5-fold increase. This situation does not predict that much of a cellular activity level difference between ICM and trophectoderm will be established. Table 10 (see page 81) shows the results of this study.

The whole embryo and ICM B6.CBA and SWR/J values have been adjusted.

Table 9 Percentage of blastocyst stage embryos with time for various strains of mice

Hours post A.I.	Strain					
	B6	CBA/J	C3H/HeHa	C3H/St	SWR/J	Ha/ICR
72	0% (3) ^a	-	0% (1)	0% (2)	0% (1)	0% (2)
74	7% (2)	-	-	-	-	13% (1)
78-80	47% (1)	-	15% (2)	24% (1)	-	60% (1)
84-86	75% (2)	30% (6)	66% (1)	70% (2)	100% (1)	66% (3)
88-90	-	-	50% (1)	75% (2)	75% (1)	100% (3)
92-94	-	-	100% (2)	-	100% (1)	-
96	100% (2)	100% (1)	100% (1)	-	-	100% (2)

^aNumber of experiments.

Table 10 Distribution of β -galactosidase activity between inside and outside cells after cavitation

Strain	Cell numbers		β -gal. activity		Estimated activity/cell ^a	
	Whole embryo	ICM	Whole embryo	ICM	Trophectoderm ^b	ICM
B6.CBA	36 [±] 3.3 ^c	19.1 [±] 0.9	2.7 [±] 0.6 ^d	1.1 [±] 0.2	1.0 ^e	0.6
SWR/J	53.1 [±] 4.9	21.4 [±] 2.51	4.2 [±] 0.51	1.7 [±] 0.13	0.8	0.8
C3H/St	49.9 [±] 4.8	20.2 [±] 2.3	4.3 [±] 0.54	0.85 [±] 0.13	1.2	0.4
C3H/St	48.8 [±] 5.4	16.1 [±] 2.2	3.4 [±] 0.63	0.63 [±] 0.75	0.8	0.4
B10.C	50 ^f	13.0 [±] 3.0	10.0 [±] 1.86	3.6 [±] 0.88	1.9	2.2
C3H/HeHa	55	20.2 [±] 1.5	5.2 [±] 0.51	2.2 [±] 0.22	0.9	1.1

^aCalculated from tabular data.

^bCell number in trophectoderm derived by subtraction of ICM counts from whole embryos.

^cStandard error of the mean.

^d $\times 10^{13}$ mol/hr.

^e $\times 10^{14}$ mol/hr.

^fFrom another experiment.

The experiments with these two strains were done with a different batch of substrate and 4-MU standard than the remaining strains. Oocytes assayed along with B6.CBA and SWR/J had twice the activity levels than when measured with later preparations of substrate and standard. For any one preparation of substrate and standard, activity levels of oocytes and embryos were reproducible (see C3H/St in table 10).

The inbred strain showing the largest difference in the cellular activity between ICM and trophectoderm is C3H/St. The difference was reproducible in two experiments with a ratio of 2-3:1 (trophectoderm vs. ICM). The data show β -galactosidase expression probably occurs in all cells of the embryo with approximately equal expression for cells of the ICM and trophectoderm. So, differences between strains for total cell per embryo is a sufficient estimate of developmental variation.

Distinctive classes of ICM activity levels were not observed among inbred strains. However, two classes of β -galactosidase activity levels may occur between strains in trophectoderm. These classes would consist of the B10.C strain with high activity levels and the remainder of the strains with trophectoderm activities about one-half that of B10.C. Neither the ICM nor the trophectoderm activity level classes agree with Bgl-s variation as the source of interstrain differences.

Timing of the Activity Level Increase

Timing of the initial increase in activity seems to be a function of when a strain's embryos cleave to the four-cell stage. C3H/HeHa embryos cleaved to four-cell faster than other strains, and these four-cell embryos had increased activities (see figs. 10-14). SWR/J embryos

initiated cleavage to the four-cell stage at the same as the other strains but had a protracted cleavage. This seems to be correlated with the delay in whole embryo β -galactosidase activity level increases relative to other strains (see figs. 9 and 12).

[Bgl] Genetic Complex and Activity Levels in Oocytes

β -Galactosidase activity levels in oocytes are influenced by [Bgl] complex variation. Substitution of the [Bgl]^{D2} haplotype into strain B6 in place of the [Bgl]^{B6} haplotype results in a halving of the ovulated egg levels (see table 1, page 85, B6 vs. B6.CBA; figs. 10 and 11). The activity level difference is statistically significant (Student's t-test, $p < 0.05$). Although the comparison between B6 and B6.CBA eggs shows a distinct effect of [Bgl] on activity levels, there appears to be no concordance between other strain egg values and [Bgl] haplotypes. The chief discrepancy on examination of table 11 seems to be in the C57BL strains (B6, B10.C and B6.CBA). The [Bgl]^C strains, C3H/HeHa and BALB/cJ, show 1.5 to 1.8-fold higher egg levels of β -galactosidase relative to the [Bgl]^{D2} strain egg levels, as one would predict because of Bgl-s regulatory allele differences but, B10.C and B6 don't have the expected high value. Neither does B6.CBA have CBA/J-like values. Three possibilities were considered to explain the discrepancy:

1. The enzyme causing the discrepancy was absorbed by the egg surface and not intracellular.
2. C57BL strains produced small eggs relative to other strains.
3. C57BL strain eggs have lower protein synthesis rates than other strains.

There is no detectable surface enzyme. That is, eggs not frozen and thawed don't show activity. The diameters of B10.C, B6.CBA and B6 eggs show no differences from the other strains. The last possibility was not directly tested. However, eggs were assayed for other enzyme activities (see table 11). α -Galactosidase activity levels in C57BL strains are less than in the other strains and the differences are significant. However, BALB/cJ doesn't have the same activity levels as C3H/HeHa as one would predict from the β -galactosidase levels. Glucuronidase activity levels show variations predicted by the [Gus] regulatory alleles in those strains. The magnitude of the difference between B6 and C3H/HeHa is similar to the difference in specific activity for adult tissues between these strains (Paigen, 1961). None of the strains showed anomalies for ovulation time or numbers of ova shed in response to PMS and HCG.

Table 11 Lysosomal enzyme activity levels of mouse eggs upon ovulation

Strain	Mean enzyme activity/egg		
	β -galactosidase	α -galactosidase	β -glucuronidase
B6	$3.52^a \pm 0.11^b (42)^c$	$1.0^d \pm 0.15 (32)$	$4.39^e \pm 0.37 (19)$
B6.CBA	$1.75 \pm 0.12 (14)$	-	-
B10.C	$3.68 \pm 0.21 (10)$	$0.86 \pm 0.1 (16)$	$5.69 \pm 0.5 (11)$
C3H/HeHa	$5.67 \pm 0.23 (18)$	$3.08 \pm 0.24 (29)$	$0.78 \pm 0.14 (12)$
BALB/cJ	$5.15 \pm 0.34 (18)$	$1.60 \pm 0.15 (9)$	$6.01 \pm 0.07 (19)$
C3H/St	$3.34 \pm 0.17 (39)$	$3.29 \pm 0.32 (13)$	-
SWR/J	$3.08 \pm 0.24 (19)$	-	-
CBA/J	$3.33 \pm 0.24 (11)$	$3.40 \pm 0.5 (13)$	$1.20 \pm 0.21 (13)$

^a $\times 10^{14}$ mol/hr/egg

^b S.E.M.

^c Number of eggs

^d $\times 10^{14}$ mol/hr/egg

^e $\times 10^{15}$ mol/hr/egg

DISCUSSION

[Bg1] Complex Control Over Embryonic β -Galactosidase Expression

The data on F_1 embryo activity level variation show that the embryonic genotype determines β -galactosidase levels beginning at the four-cell stage. This can be interpreted to mean that the structural gene is transcribed before the four-cell stage. This interpretation is strengthened by the extent of the β -galactosidase activity level increase during preimplantation and the [Bg1] complex's control over rates of the structural gene product synthesis in adult tissues. Since the amount of the activity level change is very large, it is doubtful that a stored sperm messenger RNA satisfactorily explains the paternal effect on activity levels.

Evidence of an effect by the paternally-derived allele on expression of enzymes and surface antigens prior to eight-cell stage is now documented in three cases. For glucosæphosphate isomerase (Chapman, Whitten and Ruddle, 1971; Brinster, 1973) and non-H-2 alloantigens (Muggleton-Harris and Johnson, 1976), this involved detection of the paternally-derived molecule. For β -glucuronidase, both the presence of the paternally-derived enzyme and an effect on enzyme levels by regulatory elements of the [Gus] complex has been demonstrated (Wudl and Chapman, 1976; Chapman *et al.*, 1976). Coordinate expression of β -galactosidase and β -glucuronidase in neonate mouse tissues and the similarity of the regulatory sites that affect these coordinate changes more-or-less suggest that β -galactosidase

and β -glucuronidase might have similar expression in the implantation mouse embryo as was found.

The paternal allele expression experiments support the interpretation of DNA-dependent RNA synthesis inhibitor studies that RNA synthesis, and in particular, messenger RNA synthesis, begins early and affects enzyme and antigen expression in preimplantation stage embryos. The use of DNA-dependent RNA synthesis inhibitors and two-dimensional gel electrophoresis of newly-synthesized proteins, in concert, may further elucidate what embryonic functions are controlled by synthesis of proteins from maternally-derived messenger RNA and that messenger RNA synthesized in the embryo (Levinson et al., 1978). Generalizing from the specific cases represented in paternal expression experiments, such functions might involve control of protein and lipid turnover, carbohydrate metabolism and either intercellular adhesion or cell specific recognition. The importance of this last area has been emphasized in recent research on preimplantation embryos (Ducibella et al., 1977; Kemler et al., 1977; Graham and Lehtonen, 1979). The new techniques of manufacturing monospecific antibodies will probably cause an increase in efforts to define a role of surface antigens in early development.

The β -galactosidase activity assayed in preimplantation mouse embryos shows variation in levels between B6, B6.CBA and F₁ embryos analogous to the [Bgl] complex regulatory allele variation seen in neonate and adult mouse tissues. This is true for both the ranking

of these classes of embryos for the amount of activity and the quantity of the variation. The assay itself is selective for the product of the [Bgl] complex in adult mice. The congenics and the F_1 embryos derived from them should, in theory, differ at the [Bgl] complex. Extending the analogy between adult mouse tissue variation and cleavage stage embryo variation one might expect that the phenotypic differences between the embryos of the congenics and the F_1 s are due to regulatory alleles that affect rates of synthesis over a two to four-fold range. If rates of synthesis are the only source of variation, then activity levels will vary in proportion to the rates.

A remaining question is whether Bgl-s or other genetic sites are the source of variation between strains for β -galactosidase expression in embryos. A demonstration that all β -galactosidase synthesis is subject to Bgl-s control would suggest an integral relationship between the structural gene and this regulatory element. For instance, Bgl-s differences may indicate a gene duplication. Other possibilities put the Bgl-s site in the mRNA or in the actual peptide coding sequence. Also, Bgl-s acts cis on Bgl-e expression. The cis action of Bgl-s would preclude that some sperm factor acts on an ovum-derived maternal messenger RNA or protein.

The comparison of rates of the activity level increase and activity levels between B6, B6.CBA and the F_1 derived from these two strains suggest that the level of variation is about 2.8 to 4-fold. Adult tissue activity level variation for Bgl-s is 1.8 to 2.5-fold. The magnitude of the difference between the embryos of B6 and B6.CBA is ambiguous. It could indicate Bgl-s variation is responsible for

the difference, or it may indicate the additional action of a regulatory element like Bgl-t.

Between strain variation caused by rate of synthesis differences due to Bgl-s variation would predict only two activity level classes (Felton, Meisler and Palgen, 1974). The between strain variation for cellular activity levels (see tables 7 and 8) suggest three activity level classes. The assignment of these classes is prejudiced by the [Bgl] complex haplotypes of the strains. There is no easy way to determine the extent of genetic differences between these strains that could affect β -galactosidase levels in preimplantation embryos by alteration of rates of synthesis or by other means.

When the strains are grouped by Bgl-s alleles, activity levels for ICM and trophectoderm do not fall into two classes accordingly (see table 10). First, B6.CBA should probably not be used in this comparison. Its low cell number and higher proportion of inside cells probably mean this sample was developmentally retarded compared to the rest of the strain samples (Handyside, 1978). It would have been desirable to compare B6.CBA and another C57BL strain's (B6, B10 or B10.C) embryos in order to obtain an estimate of [Bgl] complex effects on ICM and trophectoderm activity level variation. But, the B6.CBA sample was collected before it was realized that B6.CBA embryos cleave slower than other C57BL strain embryos. The two remaining [Bgl]^{D2} strains, SWR/J and C3H/St, show a surprising difference in activity between ICM and trophectoderm. This result could mean that the [Bgl] complex is not the sole source of genetic var-

iation for β -galactosidase activity levels during the differentiation of trophectoderm and ICM.

In summary:

(A) from B6, B6.CBA and F_1 embryos derived from crosses of the two inbred strains, it appears that: (1) $[Bgl]^{B6}$ and $[Bgl]^{D2}$ complex differences cause a 2.5 to 4-fold difference in β -galactosidase activity levels by 84 hours post-insemination. (2) the embryonic $[Bgl]$ complex haplotype determines the 84-hour activity level rather than the maternal haplotype. (3) the amount of variation in levels suggest that Bgl-s and some other element of $[Bgl]$ cause the difference.

(B) from the remaining interstrain comparisons, it appears that: (1) grouping the strains by $[Bgl]$ haplotype does account for some of the variation between strains. (2) this grouping of strains into three classes again suggests that Bgl-s is acting to cause some of the variation. In this instance, all $[Bgl]^{D2}$ strains have lower cellular activities than the $[Bgl]^C$ strains, but Bgl-s differences can't account for all the variation as $[Bgl]^C$ and $[Bgl]^{B6}$ strains differ in levels too. (3) this could mean that other elements in the $[Bgl]$ complex are acting or that loci other than $[Bgl]$ also cause between strain variation.

(C) from the ICM, trophectoderm interstrain comparison, it appears that: (1) the differentiation event results in cell type specific variation for activity levels in some strains but not in others. (2) Since cell specific variation occurs between strains of the $[Bgl]^{D2}$ haplotype, this suggests that Bgl-s and the $[Bgl]$ complex in general

is not the sole source of genetic variation between strains even at the cellular level.

The final determination of preimplantation embryonic β -galactosidase activity levels is probably a multigenic event analogous to the way that liver β -galactosidase levels are determined in neonate and adult mice. The action of Bgl-s is indicated by the data, but this is not conclusive.

Oocyte β -Galactosidase Activity

The results on oocyte β -galactosidase interstrain activity level comparisons indicate a tissue- and possibly a cell-specific genetic variation. This variation doesn't show complete concordance with Bgl-s variation in adult mice.

Gonadal tissues often show the expression of new isozymes or regulatory phenotypes not found in somatic tissues. Testes express isozymes coded by LDH-c and PGK-2 loci, neither of which is expressed in somatic tissues (VandeBerg, Cooper and Close, 1973; Zinkham, 1968; Lerum and Goldberg, 1974; Blanco and Zinkham, 1963). Erickson (1976) surveyed mouse strains for variation in sperm β -glucuronidase activity levels. The variation in sperm levels was not in accord with variation in other tissues. Mouse ova glucosephosphate isomerase-1 levels are under the control of a regulatory element called Org (Peterson and Wong, 1978). The site cosegregates with the structural gene and acts cis to regulate the amount of the electrophoretic form it is segregating with. The effect of the polymorphism at this site is to make more of one electrophoretic form of the enzyme than another in heterozygous

mouse ova. This form of regulation is distinct from regulation in somatic tissues where equal amounts of the electrophoretic variants are produced.

The indication of an ovum specific type of regulation for β -galactosidase, in addition to the action of Bgl-s, is analogous to the action of Bgl-t in mouse liver. Dewey and Mintz (1978) reported that some additional somatic tissues in mouse don't show interstrain differences for activity levels in accord with Bgl-s allele variation. It may be that β -galactosidase expression is controlled by a large number of genes with specific sets acting during and after differentiation in a number of organs and tissues.

β -Galactosidase Activity in Preimplantation Mouse Embryos

The activity levels of β -galactosidase of preimplantation stage mouse embryos are ten-fold higher than those reported for β -glucuronidase (Chapman et al., 1976; Wudl and Chapman, 1976) and approximately the same as those reported for α -galactosidase (Adler, West and Chapman, 1977). All three enzyme activities were assayed in ovulated oocytes, and as reported in the results section, showed a similar quantitative relation (table 11). The general pattern of activity level change with cleavage is similar for these three activities. β -Glucuronidase activity increases three-fold by sixty hours after insemination, then undergoes a seventy to one hundred-fold increase by eighty-four hours after insemination. α -Galactosidase activity levels increase more rapidly than β -galactosidase until fifty hours post-insemination

when its rate of increase declines to the same rate as β -galactosidase. The changes in α -galactosidase accumulation rates may be a consequence of X-chromosome dosage compensation (Adler, West and Chapman, 1977; Chapman, West and Adler, 1978; Epstein et al., 1978; Kratzer and Gartler, 1978; Monk and Harper, 1978).

The large increases in the activities of three lysosomal enzymes open the possibility that lysosomogenesis is going on during cleavage. A report on rat embryogenesis (Dvorak, Travník and Stanková, 1977) and one on mouse embryogenesis (Glanguzza and Mulnard, 1972) claim that the number of secondary lysosomes and the size of the organelles increases noticeably at the eight-cell stage. Of these two reports, the one on mouse embryos is far less detailed. Cytoplasmic bodies identified as secondary lysosomes and agranular endoplasmic reticulum in these studies stain for acid phosphatase activity, a marker enzyme for lysosomes (Izquierdo, 1977). Mulnard (1965) and Solter, Damjanov and Skreb (1973) first detected acid phosphatase activity at the eight-cell stage in mouse embryos. Ishiyama and Izquierdo (1977), using a similar staining technique as Solter, Damjanov and Skreb, detected the activity at all stages. The reason for the different findings between these groups hasn't been advanced. The available literature on acid phosphatase and on lysosomes in mouse embryos doesn't provide a clear picture of the organelle's genesis or its distribution between trophectoderm and ICM. In rat embryos, a quantitative analysis of lysosomes was

performed by Dvorak, Travník and Stanková. They found that the cytoplasmic density of multivesicular bodies and agranular endoplasmic reticulum decreased beginning with the first cleavage while the density of secondary lysosomes and granular endoplasmic reticulum increased. The sharpest increases occurred at the two to four-cell stage and four to eight-cell stage. The authors speculate that multivesicular bodies and agranular endoplasmic reticulum are precursors to secondary lysosomes. My data on β -galactosidase are in general agreement with the changes in secondary lysosome density seen in the rat embryo.

One can postulate an active role for secondary lysosomes in preimplantation stage embryogenesis. Protein synthesis increases throughout preimplantation, but total protein doesn't increase. The amount of protein synthesis per day is a significant proportion of the total cytoplasmic protein (Brinster, Wiebold, and Brunner, 1976; Epstein, 1975). Brinster, Wiebold and Brunner (1976) measured turnover of protein in embryos. Their data showed two classes of proteins. About forty percent had a half-life of twelve to eighteen hours and the rest had a much longer half-life. Agranular endoplasmic reticulum may store lysosomal enzyme precursors and serve as a pool for rough endoplasmic reticulum. At the four to eight-cell stage, protein synthesis begins to increase at a large rate in parallel with the appearance of rough endoplasmic reticulum. Coordinately, secondary lysosomes may be mobilized to establish higher

degradation rates to balance the changes in protein synthesis.

The cell surface of the embryo also undergoes changes with cleavage (Artzt et al., 1973; Calarco and Epstein, 1973; Ducibella and Anderson, 1975; Ducibella et al., 1975; Gooding, Hsu and Edidin, 1976; Kemler et al., 1977; Solter and Knowles, 1978; Wiley and Calarco, 1975). This includes the appearance and loss of surface antigens of both a protein and lipid nature. These changes may involve β -galactosidase more directly. Many lysosomal enzyme deficiency diseases result in storage disorders of mucopolysaccharides, sphingolipids and glycolipids (Neufeld, Lim and Shapiro, 1975). In humans, the β -galactosidase enzyme with activity toward artificial substrates shows activity toward GM₁ gangliosides (Ho, Cheetham and Robinson, 1973; Norden, Tennant and O'Brien, 1974). Deficiency diseases of this enzyme are characterized by an accumulation of glycoprotein and GM₁. Enders and Schlafke (1974) detected a thin mucopolysaccharide coat on the embryo cell surface. Pinsker and Mintz (1973) found changes in surface carbohydrate content of late stage embryos. "Lectin" glycoprotein appears on the surface of the endoderm cells in the inner cell mass (Zetter and Martin, 1978; Wartiovaara, Leivo and Vaheri, 1979). A glycolipid, the Forssman antigen, is detectable on embryonic cell surfaces at the early blastocyst stage, then disappears from the trophectoderm surface by the time the embryo hatches from the zona pellucida (Willson and Stern, 1978). The role of membrane turnover or of lysosomes in membrane turnover for cleavage stage embryos is not known.

A second role for lysosomal enzymes could be shedding the zona pellucida. There are many reports of uterine factors that have zona-lytic activity (Rosenfeld and Joshi, 1977; Mintz, 1970; Domon, Pinsker and Mintz, 1973). In situ, embryonic factors may make little contribution. The embryo can also shed its zona in vitro. Part of the process involves hatching with the embryo contorting itself and rupturing the zona (Cole, 1967). Enders and Schlafke (1965) found no evidence of sublytic digestion of the zona from inside or evidence of enzyme discharge in an electron microscopic examination of murine blastocysts. So, the role of sublytic changes in the zona is not established for in vitro shedding.

For lysosomal enzymes to participate in zona lysis, they would have to be secreted. Loss of lysosomal enzymes to the extracellular space is known (Paigen and Peterson, 1978; Meisler, 1978; Thomas et al., 1973; Glaser, McAlister and Sly, 1974; Wiseman, Vessella and Herschkowitz, 1971) in both normal and pathological processes. The leakage of lysosomal enzymes from fibroblast in "I-cell" disease is thought to involve changes in the carbohydrate moiety of these glycoproteins (Hickman, Shapiro and Neufeld, 1974). Secretion of lysosomal enzymes could occur by a discharge of lysosomes or by altering their carbohydrate content. It is doubtful that β -galactosidase would have any role in zona lysis, since its pH range is too low. Murine β -glucuronidase does have a broad pH optimum and is active at neutral pH. The concentration of β -galactosidase activity in the ovarian bursa of the rat increases at ovulation (Parr, 1974). Here

again the pH range of the enzyme is too low to suggest an active role in follicle rupture, but it may be a marker of lysosomal enzyme participation in the event. The contribution of embryonic enzyme to the implantation process is not clear either (Abraham et al., 1970).

Embryonic Cell Numbers and ICM Cell Numbers

There is a good deal of variation for cleavage rates among the strains used. Certainly if these strains cleaved at the same rate, the overall analysis of [Bg1] expression would be simplified. Cleavage rate variation among inbred strains has been noted in other work (Whitten and Dagg, 1961; Wakasugi and Morita, 1977). In general, the cell numbers per embryo found in my data agree with other studies (Chapman et al., 1976; Adler, West and Chapman, 1977; Wakasugi and Morita, 1977; Burgoyne and Ducibella, 1977; Handyside, 1978; Smith and McLaren, 1977).

There is a slight problem with the comparison between B6 and B6.CBA. Ideally, the use of a strain and its congenic partner should eliminate all genetic differences except for those on the 10 centi-Morgan stretch around the selective marker theory predicts will be carried along. However, the comparison of cell numbers between B6 and B6.CBA shows a significant difference. Artificial insemination of the females should have eliminated variation for fertilization time. The difference between B6 and B6.CBA is probably in the cleavage rate of the embryos. The donor strain for the [Bg1]^{D2} haplotype in B6.CBA, CBA/J, has the slowest rate of cleavage among the inbred strains examined. It is conceivable that the cleavage

rate difference between B6 and CBA/J is specified by a few genes. If this were the case and if one of the genes were linked to [Bgl], this might explain the result. Also, it is possible that the back-crossing of heterozygous Bgl-s^{h/d} mice to B6 did not eliminate all unlinked CBA/J genes.

The number of inside cells in embryos has been estimated by Handyside (1978) and Barlow, Owen and Graham (1972). The latter estimated that fifteen to thirty percent of the cells from embryos with total cell numbers from seventeen to sixty cells are inside cells. Handyside's estimate is double that of Barlow, Owen and Graham. Barlow, Owen and Graham were using thin sections of embryos to make their estimates while Handyside made cell counts of isolated ICMs. My data should be compared to Handyside's. In general, my counts of cell number in ICMs agree with Handyside. Some samples means show a low ICM cell count. This could be due to damage to these embryos during zona pellucida removal. The tight junctions of the trophectoderm might then have leaked antibody and complement into the segmentation cavity during immunosurgery permitting lysis of inside cells.

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